KU Leuven
Biomedical Sciences Group
Faculty of Medecine
Department of Oncology

THE MOLECULAR PATHOGENESIS OF HEAD AND NECK CANCER

THE ROLE OF TYROSINE KINASES AND HPV

Evert VAN LIMBERGEN

Jury:
Promoter: Prof. Sandra Nuyts
Co-promoter: Prof. Jan Cools
Chair: Prof. Maarten Naesens
Secretary: Prof. Vincent Vander Poorten
Jury members: Prof. Esther Hauben
Prof. Eric Deutsch
Prof. Jan Bussink

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical sciences

Met opmaak: Engels (Verenigde Staten)

February 2014

Table of contents

Abbreviations

AXL	AnneX-eLectro receptor tyrosine kinase
BCA	Bicinchoninic Acid
BMX	Bone Marrow tyrosine kinase coded in chromosome X
BOT	Base Of Tongue
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitor
CT-scan	Computer Tomography scan
CUL2 complex	Cullin 2 Ubiquitin Ligase Complex
DAB	DiAminoBenzidine
DFS	Disease Free Survival
DNA	DeoxyriboNucleic Acid
E1-7	Early genes 1 to 7 expressed by Human Papilloma Virus
ECL	Electrogenerated ChemiLuminescence
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal Growth Factor Receptor
E6AP	E6 associated protein
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence Assisted Cell Sorting
FDA	Food and Drug Administration
FFPE	Formalin Fixed Paraffin Embedded
FDG-PET	2-[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography
FLK	Fetal Liver Kinase
FLT1-3	*Fms*-Like Tyrosine kinase 1 or -3
GAPDH	GlycerAldehyde-3-Phosphate-DeHydrogenase
GFP	Green Fluorescent protein
H&E	Hematoxylin and Eosine
HGFR	Hepatocyte Growth Factor receptor
HRE	Hypoxia Response Elements
HRP	Horse Radish Peroxidase
HIF1	Hypoxia inducible factor 1 (consisting of α and β subunits)
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HR	Hazard Rate
hTERT	human Telomerase Reverse Transcriptase
IC50	Half maximal inhibitory concentration
IDH2	Isocitrate Dehydrogenase 2
IGF-1R	Insulin like Growth Factor-1 Receptor
INF-γ	Interferon-gamma
IHC	ImmunoHistoChemistry
IMAT	Intensity Modulated Arc Therapy
IMRT	Intensity Modulated RadioTherapy
IPW	Inverse Probability Weighting
KDR	Kinase-insert Domain Receptor

Km	Binding affinity of the metabolite
Ki	Binding affinity of the inhibitor
LCR	Long Control Region
LRC	Locoregional Control
MAPK	Mitogen Activated Protein Kinase
MET	MNNG HOS Transforming gene synonym of HGFR
MRI	Magnetic Resonance Imaging
MTT	3-[4,5-diMethylThiazol-2-yl]-2,5 diphenyl Tetrazolium bromide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRTK	Non Receptor Tyrosine Kinase
NSCLC	Non Small Cell Lung Cancer
OS	Overall Survival
PCR	Polymerase Chain Reaction
PE	Plating Efficiency
PH	Prolyl Hydroxylase
PI3K	Phophoinisitide-3 Kinase
PLGF	Placental Growth Factor
PVDF	PolyVinyliDene Fluoride
qPCR	Quantitative (real time) Polymerase Chain Reaction based assay
RB	RetinoBlastoma
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
RTOG	Radiation Therapy Oncology Group
SEER	Surveillance, Epidemiology and End Results
shRNA(mir)	Short Hairpin RNA (based on micro-RNA backbone)
siRNA	Short interfering RNA
SRB	SulphoRodamine B survival assay
TBS	Tris-Buffered Saline solution
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TSG	Tumor Suppressor Gene
TRKB	Tropomyosin-Related Kinase B
TROG	Trans Tasman Radiation Oncology group
TXK	TXK tyrosine kinase
S	Substrate
STAT	Signal Transducer and Activator of Transcription
SRB	SulfoRhodamine B
TNK1	Tyrosine Non-receptor Kinase 1
UICC	Union for International Cancer Control
VEGFA	Vascular Endothelial Growth Factor A
VEGFB	Vascular Endothelial Growth Factor B
VEGFR-1 or -2	Vascular Endothelial Growth Factor Receptor 1 or -2
VHL	Von Hippel-Lindau tumor suppressor protein
VMAT	Volumetric Modulated Arc Therapy

CHAPTER I

GENERAL INTRODUCTION

1. General aspects of Head and neck squamous cell carcinoma

1.1 Epidemiology and etiology

Head and neck cancer is a heterogeneous group of cancers arising from the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx [1]. More than 95% of these malignancies have a squamous histology and are referred to as squamous cell carcinoma [2]. These tumors, arising from the mucosal linings of the upper aerodigestive tract, characterize themselves by local invasion in the surrounding tissues, as well as spread to regional lymph nodes. Distant metastasis at initial presentation is uncommon, arising only in about 10% of patients [1]. According to GLOBOCAN 2008 it is the 7th most frequent group of cancers with a worldwide incidence of more than 600.000 new cases a year. The associated mortality rate is about 350.000 patients yearly [3]. This disease typically affects older patients in their 6th-7th decade and appears 3 times more frequently in men than females [4].

Tobacco and ethanol abuse, as well as Human papilloma virus (HPV) infections, are regarded as the most important etiological risk factors for developing Head and Neck Squamous Cell Carcinoma (HNSCC) [1,2]. A multitude of chemicals found in tobacco products, such as dibenz[a,*h*]anthracene, benzo-(a)-pyrene, 4-aminobiphenyl, acetaldehyde, catechol and benzene, have been shown to be carcinogenic indeed [5]. The route(s) by which ethanol may function to enhance oral carcinogenesis are not as clear, but a synergistic effect has been described with smoking [6,7]. It is possible that ethanol mediates as a solvent, thereby enhancing the uptake of other carcinogenic compounds, such as those retrieved from cigarette smoke [8]. In addition, acetaldehyde, the first metabolite produced during ethanol degradation, is known to be mutagenic [8].

Recently, high-risk human papillomaviruses (HPV), and in particular HPV16, are recognized as independent risk factors and are most strongly associated with oropharyngeal carcinomas [9]. American studies have shown that 40 to 65% of

oropharyngeal HNSCC could be attributed to HPV16 [10,11,12,13]. These HPV infections might be the cause of the increasing incidence of 2-3% yearly in oropharyngeal carcinoma, while the incidence of HNSCC in other subsites is decreasing due to a decrease in exposure to the classical risk factors ethanol and tobacco [14]. Patients with HPV-associated HNSCC tend to be non-smokers and non-drinkers, present with more advanced N-stage lesions, have younger age, and ultimately experience improved survival [9,13]. These findings were recently confirmed in correlative studies from the Radiation Therapy Oncology Group (RTOG) 0129 and the Trans Tasman Radiation Oncology group (TROG) 02.02 phase III trials [15,16].

Next to the higher mentioned exogenous risk factors, certain inherited disorders, such as Fanconi anaemia, Lynch syndrome or Ataxia Teleangiectasia and other a more general genetic susceptibility factors predispose to HNSCC [1,2]. Finally, also poor oral hygiene, unhealthy diet and an inactive lifestyle are linked to a higher risk of developing HNSCC [17].

1.2 Pathogenesis

HNSCC originates from epithelial cells lining the mucosal borders of the upper aerodigestive tract. The current paradigms suppose that normal epithelial cells eventually develop into tumoral cells through several intermediate steps like hyperplasia, dysplasia, and carcinoma in situ. Owing to the developments in molecular research during the past two decades, this has now been defined in molecular terms [2,18] . In HPV negative HNSCC one of the first steps leading to hyperplasia is loss of the chromosomal region 9p21 leading to the loss of the p16 tumor suppressor gene (TSG). Further mutation of the p53 TSG correlates with pre-invasive to invasive HNSCC lesions while EGFR (Epidermal Growth Factor Receptor) and cyclin D amplification is often seen in invasive stages of HNSCC [2,18]. On the other hand p16 and p53 TSG's are functionally inactivated by the viral oncoproteins E7 and E6 in HPV positive HNSCC [2].

An important concept, especially with respect to HPV negative HNSCC is the notion of 'field cancerization'. Due to exposure of the entire mucosal epithelium at the upper aerodigestive tract to carcinogens from tobacco smoke and alcohol, multiple subclinical precursor lesions will occur at different spots. These subclinical lesions remain often untreated and subsequently result in high rates of local recurrence and second primary formation [2]. In part this may be one of the reasons why HPV negative HNSCC's eventually experience lower overall survival rates.

1.3 Diagnosis and Staging

As a general rule in oncology, prompt recognition of pathological symptoms is important to diagnose HNSCC lesions as early as possible. Symptoms, however, may vary according to the different sublocalizations. Tumors arising in the hypopharynx may remain asymptomatic during a prolonged period and give rise to pain or dysphagia only later in their evolution, resulting in diagnosis at more advanced stages. Patients with vocal cord tumors, on the other hand, may quickly experience hoarseness resulting in early stage detection. Once a suspicious lesion has been discovered a surgical biopsy needs to be taken to confirm diagnosis. The cancerous lesion should be classified according to the World Health Organisation classification [19]. Once the diagnosis is confirmed tumors should be staged according to the TNM classification (UICC 7th edition) assessing tumor extent (T), Nodal involvement (N) and Metastasis (M). Staging methods assessing local tumor extend and nodal involvement include extensive clinical examination and palpation, pan-endoscopy and radiological imaging of the head and neck region with computer tomography scanning (CT-scan) or magnetic resonance imaging (MRI) . As a general rule, MRI is often the preferable staging procedure for every subsite except for laryngeal or hypopharyngeal cancers [20]. A thoracic X-ray or thoracic CT-scan is performed to rule out metastatic disease as well as second lung primaries in smoking patients. Further screening for metastasis in the liver can be done by abdominal echography or CT-scan although the incidence of liver metastasis is low [21]. The incidence of bone

metastasis is generally regarded too low to justify bone scintigraphy [22]. The role of 2-[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) in HNSCC is investigational. However there is proven added value in selected cases where nodal involvement exists but the primary tumor cannot be located, or in cases where conventional imaging yields equivocal results [23]. Several centers also perform endoscopy of the upper gastrointestinal tract to rule synchronic tumors. Accurate TNM staging has proven its value in facilitating treatment planning and clinical decision making, prognosis, evaluation of treatment results and research.

1.4 Treatment

When staging is completed treatment strategy should be discussed by a multidisciplinary team [20]. The first decision that is made is if the patient is curable or not, taking into account the tumor stage, as well as the patients age and comorbidity. If the patient is assumed to be *not* curable, the treatment strategies should predominantly pursue prolonged survival together with acceptable quality of life. Therefore major treatment related toxicity should be avoided as much as possible. In case of curative disease, much more toxic treatments are accepted in an attempt to completely eradicate the tumor in a specific patient.

The treatment of HNSCC is based on a combination of three major treatment arms being surgery, radiotherapy and chemotherapy. The choice of treatment depends primarily on tumor extend and localization [20]. For metastasized (and incurable) disease in general a systemic treatment like chemotherapy is necessary. In localized disease treatment is mainly based on the two local treatment arms: radiotherapy or surgery (with or without adjuvant chemotherapy).

1.4.1 Surgery

Surgical interventions can vary from local excision for small tumors to extended resections of primary tumors and regional lymph nodes with a safety margin. Much attention is given on organ preservation or reconstruction, to ensure an acceptable

functional outcome [1]. This is mainly possible for small, orally accessible lesions in the oral cavity, pharynx or larynx. Technical progress in the area of laser endoscopy and high resolution lenses have made microsurgical approaches possible, especially for small laryngeal tumors [1].

1.4.2 Radiotherapy

Radiotherapy is another treatment modality which is an integral part of the treatment approach for HNSCC. The aim of this approach is to sterilize all tumor cells in the irradiated area by making use of ionizing radiation. Usually a curative outcome is pursued and it is important that the damage induced to the surrounding normal tissues is avoided as much as possible [1]. Nevertheless, radiotherapy gives rise to a significant amount of damage or toxicity to these surrounding normal tissues. A distinction is made between acute (or early) toxicity which is in general transient and late (or chronic) toxicity which is *not* transient but rather slowly progressive [24]. Acute side effects are observed during or shortly after a course of radiotherapy while late side effects only manifest themselves after a latency period of at least 90 days after the onset of radiotherapy [24].

Acute transient side effects are usually seen in tissues with rapid cellular turnover like the gastrointestinal mucosa, the skin or bone marrow. In patients undergoing a radiotherapy treatment for HNSCC, oral mucositis with associated pain, oropharyngeal surinfections or eating disorders are a major problem, requiring supportive treatment [1]. Besides epidermolysis of the skin is also often seen.

Late and progressive chronic side effects are usually seen in tissues with slow cellular turnover. The pathogenesis of these side effects is complex but often these involve vascular changes, fibrosis and loss of functional cells [24]. In case of radiotherapy for HNSCC, the most important chronic and dose limiting side effects are dysphagia and xerostomia due to radiotherapy related damage to the swallowing structures and salivary glands [25]. These factors limit maximum dose delivered since they are chronic and have a severe impact on quality of life.

In early stage localized disease (UICC stage I-II), either conservative surgery or radiotherapy seem to give comparable loco-regional control according to the comparison of historical retrospective studies [20]. Unfortunately no direct comparison in randomized phase III trials exist. The choice of treatment is therefore mainly based on the expected toxicity from each treatment. For example an early stage lesion of the larynx will be irradiated to preserve vocal functions. On the other hand a lesion on the tongue or of the 'floor of mouth' can easily be resected with good functional outcome, while radiotherapy risks induction of osteoradionecrosis of the mandible [26].

For locally advanced disease (UICC stage III-IVb) treatment options are surgery with reconstruction and postoperative radiotherapy. For patient with high risk features (incomplete resection, or nodal extracapsular extension) postoperative chemoradiotherapy is advised [27,28]. However for resectable patients, where the anticipated functional outcome (or prognosis) is poor, combined radiochemotherapy is preferred. In irresectable patients combined radiochemotherapy is obviously the standard of care [20].

A possible role for induction chemotherapy has been reconsidered since the introduction of the taxane-platinum-fluorouracil combination has proven to be highly effective [29]. Although it is currently not considered as standard of care, it may be an option for organ preservation in advanced larynx and hypopharynx cancer in patients otherwise requiring total laryngectomy [20].

1.4.3 Current clinical challenges in HNSCC treatment

Treatment for HNSCC is highly complex, in part because of the variety of disease subsites, but also because of the intricate anatomy, with normal and tumoral structures often in close proximity. Surely, normal function of anatomical structures in the head and neck area are essential to make speech, chewing, swallowing and breathing possible. For this reason a lot of attention has been drawn to organ sparing therapies during the last decades. Here, radiotherapy has a very important role to

play since it has been shown that even in locally advanced HNSCC disease larynx and pharynx preservation is possible [30,31]. About 2/3 of patients are diagnosed with locally advanced disease [1]. In this group however less than 1 patient out of 2 will eventually experience long term tumor cure. The rest of patients will suffer from local or distant HNSCC relapse and will eventually succumb as a consequence of disease progression [1].

Intensification of the radiotherapy treatments used for the majority of locally advanced HNSCC's (stage III-IVb UICC) did result in significantly improved local control and survival. Several different strategies have been successfully applied, using concurrent chemotherapy, hyperfractionation or accelerated radiotherapy [32,33]. Hyperfractionated radiotherapy schedules use multiple small fractions daily and therefore limit late toxicity but not acute toxicity [24]. This allows then for a limited dose escalation leading to higher dose on the tumor, associated with better cure rates, while having comparable chronic toxicity in exchange for more severe acute toxicity. In accelerated treatment the overall treatment time is shortened without reducing total dose or fractionation size. These schedules aim to reduce tumor repopulation as a cause of radiotherapy failure .

Although these schedules improve local control and survival they come at a cost of increased toxicity [34]. Currently, no further intensification of these schedules seems possible as almost half of the surviving patients will suffer from severe long term toxicity with an associated impact on quality of life[35].

As toxicity is currently dose limiting and treatment failure remains high, new treatment strategies are needed. Novel strategies to overcome these problems involve more complex dose delivery techniques like intensity modulated radiotherapy (IMRT), Intensity Modulated Arc Therapy (IMAT), Volumetric Modulated Arc Therapy (VMAT) or Tomotherapy, allowing more steep dose gradients around the targeted areas resulting in additional sparing of the normal tissues. However, one must be

very cautious that inappropriate sparing of normal tissue could lead to avoidable marginal recurrences [25].

Another possible way to improve cure rates in advanced HNSCC makes use of smart molecular targeting strategies. Here one tries to interfere with tumor specific intracellular pathways to target tumor cells and *not* healthy cells. Subsequently these strategies could improve cure rates without adding more toxicity. Epidermal growth factor receptor (EGFR) targeting using the monoclonal antibody Cetuximab is a nice example of such a strategy. For this, the clinical benefit -in association with radiotherapy- was recently proven in a phase III clinical trial [36]. Concurrent addition of the EGFR targeting antibody Cetuximab to radiotherapy improved 5 year overall survival from 36.4% to 45.6% in patients treated for locally advanced HNSCC [36].

2. Molecular pathogenesis of HNSCC: insight reveals possible molecular targeting strategies

2.1 Definition of molecular targeted therapy

According to the dictionary of National Cancer Institute of cancer terms, molecular targeted therapy encompassed substances that kill cancer cells by targeting key molecules involved in cancer cell growth. The term refers to a new generation of cancer drugs designed to interfere with a specific target pathway that is believed to have a critical role in tumor survival, growth or progression. This approach contrasts with the conventional cytotoxic chemotherapeutics that have been used in cancer therapy in past decades. Here -in general- all rapidly dividing cells are affected resulting in well-known side effects like hair loss, mucositis, nausea and vomiting, diarrhea, neutropenia, anemia etc...

A distinction is made between direct and indirect targeting strategies as well as active and passive targeting strategies [37]. *Direct* molecular targeting strategies make use

of compounds that directly interfere with a molecular pathway (or process) that is essential for tumor cell survival. On the other hand, *indirect* targeting strategies rely on tumor antigens expressed on the cell surface, which in turn serve as target devices for compounds containing different kinds of cytotoxic effector molecules. In such an approach, drugs can *actively* bind (or target) tumor cells using tumor-specific monoclonal antibodies or peptide ligands having high affinity to specific receptors or antigens present on these tumor cells. In addition to the higher described '*active'* targeting strategies, tumors can also be '*passively targeted'* by macromolecules through the "enhanced permeability and retention effects" attributed to the hyperpermeable angiogenic tumor vasculature. In this manuscript we use the term targeted therapy only for *active direct* targeting strategies.

2.2 Seven essential characteristics of a good candidate for targeted therapy

In my opinion there are several properties required for a targeted therapy to be successful in clinical practice. First of all, the target should be expressed the tumor cells. Secondly, the target pathway should be activated in most (or preferably all) cancer (stem) cells if the pathway is an oncogenic driver pathway. Third, tumor cell survival needs to depend on the activation of the pathway. This phenomenon is called 'oncogene addiction'[38]. However if the pathway is a tumor suppressor pathway it should be inactivated in most cancer cells, and then activation should be incompatible with cell survival. Fourth, the aberrant activation (or inactivation) status of the pathway should be essential for survival of tumor cells and not essential for normal cells. As a consequence one would expect that interfering with the pathway would be much more toxic to tumor cells than normal cells, thus creating a therapeutic window. Fifth, the pathway should be drugable, allowing intervention in clinical practice. Sixth, there should be an adequate biomarker to identify which tumors do or don't have the specific oncogene addiction, allowing to select the correct group of patients for the treatment. And to finish, the higher the prevalence of tumors with this specific pathway abnormality the better, making sure the new

targeting strategy can aid many patients. Only when all these criteria are fulfilled the new treatment can have an important impact on patient care.

2.3 Molecular pathways in HNSCC

A first step into developing new targeting strategies is a profound understanding of the molecular processes that drive cancer progression. During the last decades, aberrant signaling in many pathways was uncovered. Here we will describe the most established molecular pathways, leading the development of HNSCC.

2.3.1 Aberrant tyrosine kinase signaling

The Epidermal Growth Factor Receptor (EGFR) may be the most studied member of the receptor tyrosine kinase (RTK) family (see further). Multiple studies confirm EGFR overexpression in HNSCC in up to 90% of cases [39-42]. EGFR overexpression leads to transformation of oral keratinocytes *in vitro* [43]. In addition, EGFR inhibition in HNSCC cells induces cell death and radiosensitivity *in vitro*, *in vivo* and *in clinical practice* [36,44-46]. The reasons for aberrant EGFR signaling activity in HNSCC are mostly unknown. Only very limited amount of mutations have been described [47]. Gene amplification occur in HNSCC but can only explain EGFR overexpression in 30 % of these tumors [48]. Hypoxia induced EGFR protein translation has been suggested as a possible explanation for increased EGFR overexpression in other tumors [49]. Finally, also a mutant form of EGFR has been described, referred to as EGFRvIII. This mutant, first described in glioblastoma, misses exons 2 to 7 and is constitutively active. Therefore it drives tumor progression. However the data on the prevalence of this mutated form of EGFR are conflicting as they seem to vary from 0 to 44% [40,50]. EGFR activation does lead to a plethora of downstream signaling cascades resulting in activation of downstream pathways, including the MAPK pathway, the AKT pathway, STAT and the PLCγ pathway. Also nuclear translocation of the EGFR receptor has been described as an important signaling process. By doing so EGFR affects cellular growth, resistance to apoptosis, and DNA repair[51] .

In addition to EGFR, the tyrosine kinase 'MET' has also been recognized as an important driver in HNSCC. MET influences cell viability and motility through activation of the AKT and MAPK signaling pathways. MET overactivity has been linked to gene amplification and activating mutations [52]. MET or its ligand '*hepatocyte growth factor*' have been reported to be overexpressed in up to 80% of HNSCC [53].

2.3.2 Aberrant PI3K - AKT signaling

This pathway is typically activated through receptor tyrosine kinases like EGFR or MET. These TK's subsequently activate Phosphoinisitide-3 Kinase (PI3K). PI3K then leads to activation of the serine-threonine kinase 'AKT'. Activated AKT in turn activates many other intracellular signaling cascades, eventually leading to increased proliferation, resistance to apoptosis, angiogenesis and invasion/metastasis. Therefore this pathway is established as a key oncogenic driver in HNSCC [54]. It has been shown in HNSCC cell lines, that many of the documented PI3K mutations are gain of function mutations [55]. Also deletions or loss of function mutations in the PI3K *de*activating phosphatase PTEN have been described, meaning that PI3K cannot be shut down once it gets activated [56]. A whole-exome sequencing study detected mutations in the PI3K pathway in 30% of 151 HNSCC samples [57]. These include mutations in the PI3K complex itself, the PI3K inactivating phosphatase PTEN, AKT or other associated genes of this pathway.

2.3.3 TGFβ signaling aberrations

The transforming growth factor beta (TGFβ) pathway is another important inhibitory pathway that is frequently *down*regulated in HNSCC. TGFβ signals through the TGFβ receptors. These receptors transduce their signals through phosphorylation of the SMAD-2 and -3 proteins which -after complexing with SMAD-4- regulate transcription of target genes. As a consequence, cellular proliferation and survival is decreased while apoptosis is increased [2]. In HNSCC TGFβ signaling is often abolished through loss of chromosome 18q, containing the SMAD- 2, -3 and -4 genes

as well as the TGFβ-II receptor. Also SMAD-2 and SMAD-4 mutations have been described [2,58].

2.3.4 Defects in p53 and p16-retinoblastoma signaling pathways

P16 and p53 are crucial genes involved in cell cycle. Defects in these genes result in loss of cell cycle control and give rise to limitless replicative potential. Somatic mutations of the p53 encoding gene TP53 are found in 60-80% of HNSCC [2]. The gene locus CDKN2a which encodes the p16 cell cycle inhibitor is also frequently inactivated. P16 inhibits the retinoblastoma protein (pRB) tumor suppressor through inactivation of Cyclin Dependent Kinases (CDK) -4 and -6. In HPV negative HNSCC p16 is most frequently inactivated through homozygous deletion, but inactivation through gene promotor methylation (leading to gene silencing) or mutation of the CDKN2a gene locus is also seen [59]. In HPV positive HNSCC, both p53 and p16-retinoblastoma pathways are blocked through the E6 and E7 oncoproteins in order to induce cancer (see point 4.1.2). Both p16 and p53 pathways are known to serve as a backup to each other, meaning that inactivation of both pathways seems a prerequisite for immortalization [60-62].

2.3.5 NOTCH1 pathway signaling aberrations

Only recently, whole exome sequencing studies demonstrated mutations in the NOTCH 1 gene, with a prevalence of around 15% [63,64]. After binding of 'Jagged' or 'Delta' ligands, the intracellular domain of NOTCH1 will be released after proteolytic cleavage. This intracellular domain will then alter cellular processes mediating as a nuclear transcription factor affecting gene expression [65]. However, the mechanism of how NOTCH1 is contributing to HNSCC development is not clear. On the one hand, activating mutations have been shown to contribute to the development of chronic lymphocytic and T-cell lymphoblastic leukemia's [66,67]. On the other hand, reduced NOTCH signaling seems to be involved in the development of myeloid leukemia [68]. These observations demonstrate that depending on the context, NOTCH can either act as a tumor suppressor or as an oncogene. In HNSCC, the existence of focal

deletions and nonsense mutations however primarily suggests it to act as a tumor suppressor gene in HNSCC, in contrast to T-cell lymphoblastic and chronic lymphocytic leukemia's where activating mutations were mainly found in hotspots [63,67-68]. In line with these observations it has been shown that NOTCH 1 knockout mice show a tendency to develop epithelial tumors [69]. The contribution of the NOTCH 1 pathway to the development of HNSCC however needs further study.

2.3.6 VEGFA signaling

The attraction of new blood vessels is an essential characteristic of cancer cells. Otherwise blood supply becomes insufficient to provide fresh nutrients and oxygen to a growing tumor where the distance between the newly formed tumor cells and existing blood vessels progressively increases. There are many different inducers of angiogenesis, but VEGFA is widely accepted as the key player [70]. VEGFA is therefore assumed to be an essential oncogenic driver in any type of solid tumor, including HNSCC. Moreover several studies show that increased VEGFA expression correlates with worse outcome in HNSCC [36,71,72]. The molecular basis for this is not clear, but it is reasonable to assume that tumor vascularisation and possible tumor hypoxia play a role. Interestingly a recent study showed that VEGFA also drives tumor proliferation of HNSCC cells independently of angiogenesis [73].

2.4 Current and promising target approaches

Although *EGFR* overexpression is seen in up to 90% of HNSCC, only 10 to 16% of patients benefit from EGFR targeted therapy [36,46,74,75]. Predictive markers to forecast treatment response are currently not available. The low response rates can be explained by multiple factors. First of all, only a small proportion of EGFR overexpressing HNSCC shows receptor cross phosphorylation, a sign of receptor activation [40]. Other reasons proposed are cross activation through other RTK's, the existence of the higher described EGFRvIII variant having a lower binding affinity to the EGFR targeting monoclonal antibody Cetuximab, and epithelial-to-mesenchymal transition of tumor cells [76,77]. Also activating mutations in the PI3K-AKT pathway

downstream of EGFR signal transduction may explain treatment failure. Many EGFR inhibitors are available. These inhibitors are currently grouped in monoclonal antibodies (Cetuximab, Panitumumab, Nimotuzumab, Zalutumumab) or tyrosine kinase inhibitors (TKI's) (Erlotinib, Gefitinib, Afatinib, Lapatinib, etc…)[76]. Currently only Cetuximab is FDA approved for clinical use. Cetuximab is actually the only approved targeted therapy in HNSCC, and the only one that is currently known to enhance radiotherapy response in clinical practice [36]. The enhanced radiotherapy response through EGFR inhibition can be explained through decreased DNA repair and *de*activation of the AKT and MAPK survival pathways leading to increased apoptosis following (chemo-)radiation induced DNA damage [51] .

MET is another promising target in HNSCC treatment, as previously discussed. Preclinical evidence shows that targeting of the MET receptor leads to better treatment responses. These data show a synergic effect when combined with other cytotoxic drugs [52]. The interaction of MET inhibition combined with radiotherapy is not described. Several MET inhibitors, such as Foretinib, are in advanced stages of clinical development [76].

The *PI3K – AKT pathway* is overactive in HNSCC, either through aberrant RTK activation or though mutations resulting in pathway overactivity (see point 2.3.2). Several inhibitors are in development, like the PI3K inhibitor NVP-BEZ235, or the AKT inhibitor MK-2206. In general early clinical trials show (moderate) effectiveness of these drugs, and demonstrate they are well tolerated [78]. Activation of the PI3K/AKT/mTOR pathway is implicated in all major mechanisms of radioresistance, including intrinsic radioresistance, tumor cell proliferation, and hypoxia [79]. Results from a preclinical study show that inhibition of the PI3K pathway can enhance radiosensitivity, certainly in p53 wild type tumors [80]. Thus, blocking the PI3K/AKT/mTOR pathway has great potential to enhance the effectiveness of radiotherapy for HNSCC patients.

Only recently it was discovered that mutations in the *NOTCH1* pathway are frequently associated with HNSCC [63,64]. However, first, a better understanding of the mechanisms which contribute to HNSCC development is needed before targeting strategies can be formulated.

Due to the prevalence as well as the critical nature of TP53 mutations in solid tumors (including HNSCC), reconstitution of the p53 pathway have been a subject of intense investigation. Several compounds with the ability to restore mutated p53 function have been developed (e.g. PRIMA-1, CP-31398). These compounds have shown to be effective in restoring p53 function as well as induction of apoptosis in HNSCC cell lines [81]. A PRIMA-1 derivate, PRIMA-1^{MET} has recently been tested in a phase I clinical trial where the compound was not only found to be save but was also found to be effective in restoring p53 function in human patients [82]. Since mutated p53 has been associated with radioresistance *in vitro* as well as *in vivo*, these compounds also hold promise as to enhance radiotherapy response in HNSCC [83]. Another interesting strategy to restore p53 function in HPV-positive HNSCC is the use of proteasome inhibitors, which prevent virally mediated p53 degradation. A phase I clinical trial was attempted using the proteasome inhibitor bortesomib together with radiotherapy and cetuximab. However this trial was prematurely terminated as 5 out of 6 patients progressed. Correlative studies proposed that upregulation of EGFR secondary to decreased proteasomal degradation rendered these HPV positive tumors more radioresistant [84].

3. Tyrosine kinases as attractive targets in oncology

3.1 What are tyrosine kinases?

Tyrosine phosphorylation has emerged as a fundamentally important mechanism of signal transduction and regulation in eukaryotic cells, allowing cells to appropriately respond to many intracellular or extracellular environmental changes. Perturbations in tyrosine phosphorylation underlie many human diseases, in particular cancer. The tyrosine phosphorylation reaction is catalyzed by a specific subset of the protein

kinase family: the tyrosine kinases (TK's) [85]. The TK family consists of 90 different proteins, which are further classified into 58 transmembrane receptor type TKs (RTKs) and 32 intracellular non-receptor type TK's (NRTKs). RTK's consist of an N-terminal region which contains in general a ligand binding domain and a single transmembrane helix to anchor the protein into the plasma membrane. An intracellular signal transduction domain, contains an enzymatic tyrosine kinase domain as well as multiple tyrosine residues which, when phosphorylated, enhance receptor catalytic activity or provide docking sites for downstream signaling proteins [86,87]. NRTKs lack ligand binding and transmembrane domains but contain a variety of domains that mediate interactions with other proteins, lipids and DNA. They are found in the cytosol, the nucleus, and the inner surface of the plasma membrane. Tyrosine kinase activity is strictly regulated, and activation typically requires dimerisation and *trans*-phosphorylation [88]. Activated TK's can then affect cellular behavior through several downstream signaling cascades like the PI3K-AKT pathway, the MAPK pathway, the '*signal transducer and activator of transcription'* (STAT) and PLCγ-pathway.

3.2 Why tyrosine kinases are interesting targets in head and neck squamous cell carcinoma

Tyrosine kinases have been known to regulate many intracellular processes in mammalian cells [89]. However the true depth of the whole signaling system only came apparent recently. Mass spectrometry-based phosphoproteomic analyses for phosphorylated tyrosine residue containing peptides in different cell types and tissues have revealed hundreds of tyrosine phosphorylation sites to be occupied in established proteins involved in intracellular signaling, also proteins involved with DNA synthesis and processing, energy and metabolism, immunity, storage and transport, protein synthesis were identified. On top of that deregulated function is

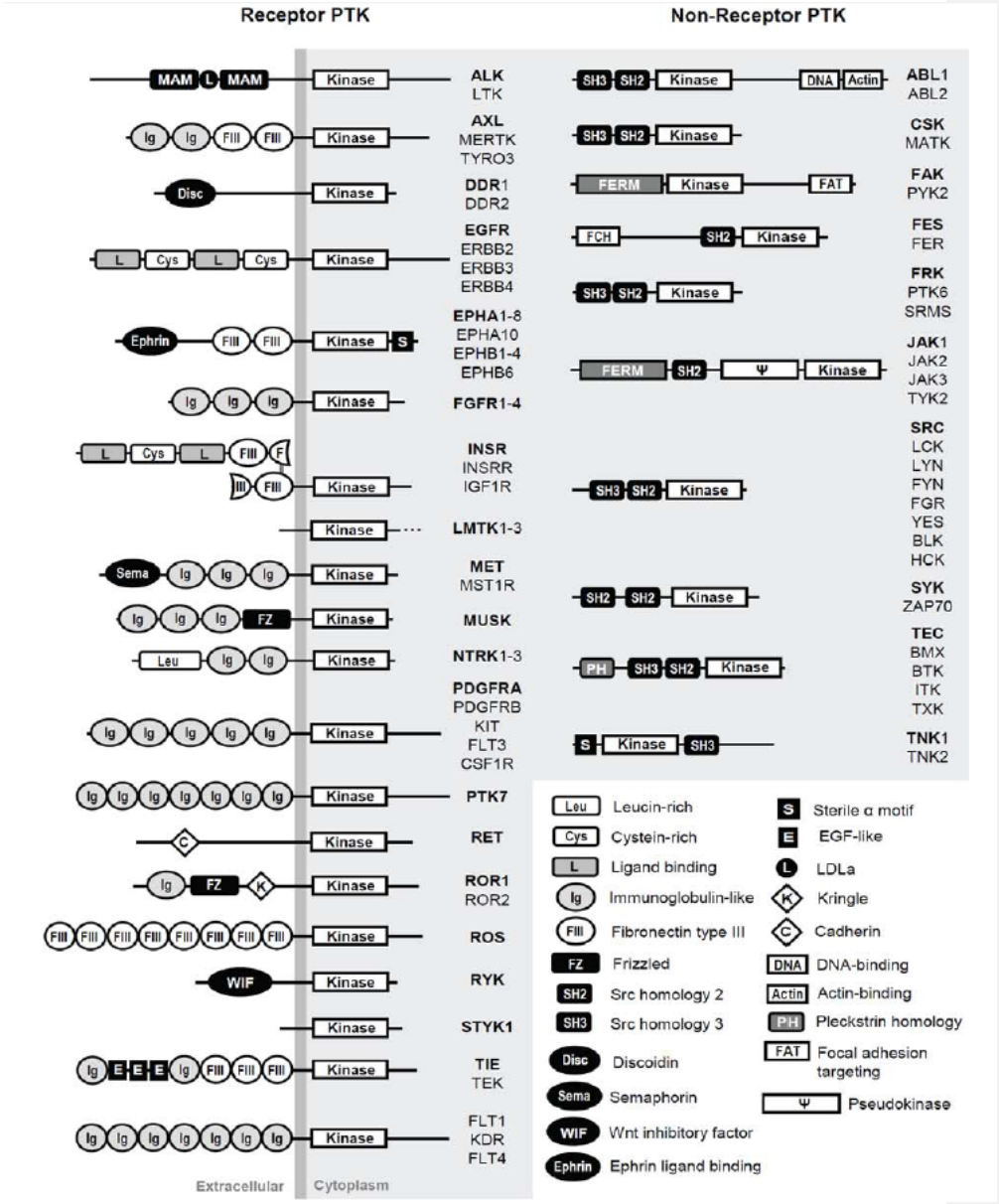


Figure 1: *Overview of the classification and global architecture of the 90 human protein tyrosine kinases.PTK families are indicated in bold.*
Adapted from Porcu. Protein tyrosine kinase signaling in T-cell acute lymphoblastic leukemia, 2012

typically seen in many cancers. For at least 51 of 90 tyrosine kinases, an active role in oncogenesis has already been described in various tumor types [90].Among these, both increased and decreased TK activity has been shown to be important. Many different mechanisms have been described leading to aberrant TK activity, like constitutively active TK fusion proteins, TK activating mutations, overexpression and downregulation [90,91].

For many of these TK's direct targeting strategies exist. Monoclonal antibodies, with receptor blocking activities can be applied to RTK's. Theoretically, in case of oncogenic repressed TK activity, an activating ligand could be administered to upregulate signal transduction. Also, an entire class of membrane permeable small molecular tyrosine kinase inhibitors (TKI) exists showing activity against a wide variety of TK's [85,91]. Even now many new compounds are in development.

In HNSCC, aberrant activity of some tyrosine kinases has been linked to the development of HNSCC. Before 2008, the start date of this PhD thesis, the involvement of EGFR in HNSCC development, survival and resistance to radiotherapy had been well established (see 2.3.1), but also the additional role of a few other tyrosine kinases began to surface, like for example that of insulin like growth factor 1-receptor (IGF-1R) or Tropomyosin-Related Kinase B (TRKB) [92,93]). Later in 2009, the involvement of MET and TRKB kinases had been reported in the development of HNSCC. Although their role in radioresistance still remains unclear [52]. The importance of the remaining part of the extensive TK family in the development of HNSCC, as well as their contribution to radioresistance is not known.

4. Human Papilloma virus (HPV) and Head and Neck Squamous cell Carcinoma

4.1 Human papilloma viruses

The papilomaviridae are a family of polyhedral DNA-viruses. On the basis of evolutionary relationships this family is divided into different genera. The largest genera are the alpha, beta and gamma genera. The main part of the HPV family

belongs to the alpha genus. These are associated with mucosal infections. More than 100 subtypes are identified on the basis of DNA-sequence analysis. Depending on the ability of the subtype to immortalize keratinocytes and induce cancer, these HPV viruses are subdivided into high and low risk viruses [94]. The HPV-16 and 18 subtypes are the most frequently occurring high risk viruses, and are associated with the development of cancers of the cervix uteri or oropharyngeal HNSCC [95].

4.1.1 Structure of the HPV genome

HPV has a 8000 base pair long double stranded genome. The genome is divided into three regions of unequal size: the long control region (LCR), the early genes encompassing the E-1 up to E-7 genes, and the late genes (L1-L2). The L1 and L2 genes encode for the large and small capsid proteins and are only expressed in terminally differentiated epithelial cells. The E1 gene encodes for two polypeptides that bind the LCR region, thereby regulating the replication of viral DNA. The E2 protein also regulates DNA replication, but also regulates the expression of the E6 and E7 oncogenes, giving in turn rise to accelerated cell proliferation. The E4 gene is only expressed in the later phases of viral replication and regulates the maturation and release of viral particles [96]. The E5 protein seems to enhance viral genome amplification and increase the expression of the L1 and L2 genes in later phases of viral replication [97]. It also plays an important role in oncogenesis, for example by activating the EGFR receptor [98]. The E3 gene has no known product or function.

4.1.2 Life cycle of HPV and oncogenesis

HPV infection is limited to the basal cells of stratified epithelium, the only tissue in which they replicate [99]. Micro-abrasions in this epithelium expose segments of the basement membrane and allow the virus to make direct contact. In this initial phase of viral infection cell cycle progression of the infected cell seems required, as the monomeric episomal viral genome is co-replicated with that of the host cell [100]. In a second phase when the epithelial cells differentiate, viral transcription, including that of the viral E6 and E7 oncogenes, is markedly upregulated together with

Gewijzigde veldcode

vegetative DNA amplification. The assembly of new viral particles occurs only in squamous epithelia undergoing terminal differentiation when the capsid genes L1 and L2 become expressed. Ultimately, newly assembled viruses will be released together with these terminally differentiated cells [100].

The role of the viral oncogenes E5 and especially E6 and E7 was first described in cancers of the cervix uteri [101]. Viral oncogenesis starts with the integration of the viral genome into the host cell. This integration step gives rise to the destruction of the early viral E2 gene [102]. The resulting loss of control over the transcription of the E6 and E7 genes leads to overexpression of the corresponding oncogenes. The E7 oncogenes seem predominantly important in early oncogenesis, while the E6 oncogenes seem more important in later phases on tumor development [101,103].

The E6 protein fulfills its oncogenic properties mainly through downregulation and inactivation of the p53 tumor suppressor protein. Through its affinity to bind E3 ubiquitin ligase as well as E6 associated protein (E6AP) it complexes with p53 and subsequently marks it for degradation. One of the many consequences of p53 inactivation is a loss of suppression on the CDK2-cyclin E complex, through decreased expression of the Cyclin Dependent Kinase Inhibitor (CDKI) $p21^{CIP}$. As a result the cell cycle checkpoints at G1-S and G2-M become deregulated. Further, the E6 protein induces the expression of human telomerase reverse transcriptase (hTERT)[104].

The E7 protein binds the 'cullin 2 ubiquitin ligase complex' (CUL2 complex) thereby ubiquinating the pRB tumor suppressor. This, in turn, results in uncontrolled E2F-related mitogenic transcription activity leading to cell cycle progression through the G1-S checkpoint. The uncontrolled transcription activity also leads to upregulation of the p16 CDKI, in an attempt to generate negative feedback on this defect overactive signaling pathway [105]. Further E7 also interferes in part with the $p21^{CIP}$ and $p27^{KIP}$ CDKI's[104].

Eventually these processes together with other known and unknown (epi)genetic abnormalities will lead to tumor development.

4.2 The biological basis for the enhanced radiotherapy response

Human Papilloma Virus (HPV) has recently been attributed as a major etiologic cause of HNSCC located in the oropharyngeal subsite. These cancers are characterized by their good prognosis. The epidemiologic aspects of these virally related cancers have already been discussed in point 1.1. Here we will discuss the current knowledge on the biological factors determining this better treatment outcome.

4.2.1 Favorable patient characteristics

Numerous studies have shown that patients with a HPV-related HNSCC have better performance status, younger age, are lesser smokers, show less ethanol abuse, and have a better socioeconomic status [13,15,106,107]. HPV-related cancer patients also develop fewer second primary tumors, probably due to reduced field cancerization following tobacco and ethanol abuse [108-110]. Although these factors certainly have an impact on improved survival, the relative contribution of these variables is estimated only at about 10% of the total difference in prognosis [15].

Most studies that report on better treatment outcome describe patients following radio- and or chemotherapy [10,15,16,107,111]. Although better treatment outcome has also been reported in patients primarily treated with surgery, most of the patients in this study also received postoperative radiotherapy [109]. Therefore the improved outcome after surgery alone is not clear.

4.2.2 Molecular parameters associated with better (chemo)radiotherapy response

4.2.2.1 Viral oncogenes

Numerous studies have investigated the impact on E6 or E7 overexpression in cancer cell lines. Data are however conflicting. Some studies show no effect from E6 and/or E7 upregulation or downregulation [112-114]. Other studies on the other hand show increased radioresistance after upregulation of the E6 oncogene [115], or radiosensitization following downregulation of the E6 and E7 oncogenes [116,117]. It needs to be noted that all these studies, or (a), express the HPV oncogenes in tumor

cell lines showing a non-HPV-related background with numerous other molecular defects, or (b) change 'physiological' expression levels of the HPV oncogenes in HPV-related backgrounds. So, none of these studies represents the natural situation where normal oral keratinocytes are transformed by the E6 and E7 oncogenes. Perhaps this is the reason why none of the higher described data correlate to the actual clinical observations.

4.2.2.2 (Partly) functional p53 tumorsuppressor

Initial studies documented p53 mutations in HPV related HNSCC to appear together [118-120], which is in strong contrast to observations in cervical carcinoma, another HPV related cancer. In these studies HPV status was assessed using PCR only. As these assays are extremely sensitive as they can detect only a few copies of viral DNA, and may detect not only the oncogenic infections, but also productive infections, virions or even laboratory artifacts or contaminations. Therefore, detection of viral *E6* and *E7* transcripts seemed to be a more reliable assay for the detection of an oncogenic HPV infection in HNSCC than PCR amplification of HPV DNA. Indeed, when using *E6* and *E7* expression as a 'gold standard', all E6- and E7-positive cases were *TP53* wild type as expected [121,122]. The absence of disruptive p53 mutations together with an intact apoptotic response to radiation induced DNA-damage have been suggested by several authors as a possible explanation for the better outcome after radio(chemo)therapy [123,124]. Moreover p53-mutation status (classified as p53 wild type, nondisruptive and disruptive) has been shown to negatively correlate with outcome. However one must remark that these data only exist for a surgical series in which the relative contribution of radiotherapy is not reported [125]. Nonetheless, a recent study eventually confirmed this presumption by showing that p53 in not completely suppressed in HPV-positive HNSCC cancer cell lines and that complete RNA-interference mediated p53 knockdown indeed resulted in an increased radioresistance [126].

4.2.2.3 Immune system

In principle, one could expect a more efficient immune response towards HPV-positive HNSCC, owing to the expression of E6/E7-specific antigens not present in non-infected mucosa. Moreover radiotherapy seems to synergize with the immune system to eliminate tumor cells through many ways, which are already reviewed elsewhere by Vu H.L. et al [127]. In line with the expectations, studies in xenograft models of HPV positive and HPV negative tumors revealed that HPV positive tumors seemed more sensitive to radiation, especially in immune-competent mice [128].

4.2.2.4 Intrinsic radiosensitivity DNA repair defects

Although some authors did not document a difference in intrinsic sensitivity to radiation in HPV positive vs. HPV negative cell lines [129], others do [126,130-132]. Moreover, we and others managed to demonstrate specific HPV related defects in DNA repair pathways. For example defects in homologous recombination have been described [133] , a malfunction that seems to be related to E7 related p16-overexpression [130]. Further others demonstrate DNA repair defects as result from SMG-1 promoter methylation selectively in HPV related HNSCC [131]. SMG is next to ATM and ATR an important player in the DNA-damage response network [131].

4.2.2.5 Hypoxia

Decreased oxygen tensions appear in solid tumors as a result of their unarranged chaotic vasculature. The response of cells to radiotherapy is strongly dependent on oxygen [24]. As a result, tumor cells living in hypoxic regions of a tumor become radioresistant, and this hypoxia induced radioresistance has preoccupied radiation biologists for a more than a century [134]. In several studies it has been tried to influence the outcome of HNSCC using an approach combining radiotherapy and hypoxia targeted therapy [134]. Interestingly, although hypoxia targeted therapy (being nimorazole or tirapazamine) seemed to be effective in HPV negative disease, it was not in effective in HPV positive HNSCC [135,136]. Assumptions that a better radiotherapy response in HPV related HNSCC could be associated to the better tumor oxygenation were refuted as several studies showed no differences in the levels of

hypoxia between HPV positive and negative HNSCC [137,138]. Furthermore, it appears from the results of a study performed by Toustrup et al., that hypoxia does not seem to have a negative impact radiotherapy outcome [138]. To date, these intriguing findings remain unexplained on a molecular level. This seeming paradox however could be explained by the possibility of hypoxia intolerance in HPV related cells. This will be discussed in the following paragraph.

4.2.2.5.1 A possible role for p16 in hypoxia tolerance

4.2.2.5.1.1 HIF-1, an essential mediator of cellular hypoxia response

The Hypoxia inducible factor-1 (HIF1) signaling cascade is generally accepted as a key modulator responsible for mediating an adequate cellular response to oxygen deprivation. HIF1 is a heterodimer protein existing of a HIF1α and β subunit. Normally, the HIF1α subunit is rapidly degraded due to hydroxylation by prolyl hydroxylase (PH), a process requiring adequate levels of oxygen. This hydroxylated HIF1α subunit will then interact with the Von Hippel-Lindau (VHL) tumor suppressor protein, leading to ubiquitination and degradation of HIF1α. However when no oxygen is present HIF1α binds the constitutively present HIF1β subunit and subsequently translocates to the nucleus. There, the HIF1 heterodimer recognizes and binds 'hypoxia response elements' (HREs) in the genome, and alter gene transcription. These include genes allowing for (a) the production of angiogenic factors (like VEGFA) to recruit new blood supply, (b) genes allowing for anaerobe glucose metabolism, (c) genes that allow for extracellular pH regulation [139,140]. Together these changes allow (tumor)cells to survive under hypoxic conditions.

4.2.2.5.1.2 HYPOTHESIS: p16 as an HIF-1 inhibitor, and possible impact on hypoxia tolerance

In HPV positive HNSCC p16 is overexpressed as a result from the functional inactivation of pRb by E7, as a consequence of feedback loops [105]. On the other hand, p16 expression is often lost in HPV negative HNSCC [59]. Therefore p16

overexpression is often used as a surrogate marker for HPV positive disease in HNSCC [15,16,141]. It is currently assumed that p16 has no biological activity in HPV related cancer since its signaling pathway is abrogated downstream at the level of pRB [15,95,142]. However the rising evidence for the predictive strength of p16 expression in the literature allowed us to hypothesize that p16 may still have a profound role in these cancers: a) studies show p16 immunohistochemistry to predict treatment outcome better than direct HPV testing [15,16], b) HNSCC positive for HPV on PCR but lacking p16 overexpression do not show improved prognosis [143]; c) p16 overexpressing tumors with a negative HPV status are still associated with favorable prognosis [144].

We believe p16 possibly could affect hypoxia tolerance of HPV related cancer cells, and therefore explain the higher described intriguing clinical behavior of HPV-positive cells in response to hypoxia or hypoxia targeted therapy. Indeed, Zhang *et al.* has shown that p16 is able to bind HIF1α in breast cancer cells and suppress its activities as a transcription factor. Therefore it is possible that HPV positive HNSCC become intolerant to hypoxia. As HPV positive HNSCC cells do not thrive under hypoxic conditions, there would be no negative impact of hypoxia on radiotherapy related treatment outcome, and no additional benefit would be expected from hypoxia targeted therapy.

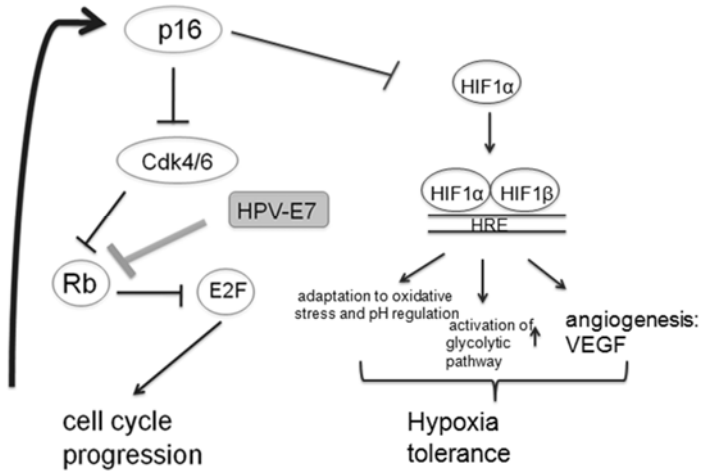


Figure 2: This scheme explains the hypothesis on how HPV related P16 overexpression could alter hypoxia tolerance in HPV positive HNSCC, by interfering with HIF1a signaling

CHAPTER II:

OBJECTIVES & AIMS

1. Objectives and aims

This PhD thesis started as two translational research parts: a 'high risk' research project investigating tyrosine kinase signaling in HNSCC and a second 'low risk' project mainly investigating the epidemiology of HPV related HNSCC in the region of Flanders.

1.1 Tyrosine kinase project in HNSCC

Tyrosine kinases are an interesting family of proteins to investigate as new therapeutics in the context of HNSCC. The clinical success of EGFR targeted therapy provides good proof of concept as one of the first examples of successful bench to bedside research [145]. However, EGFR targeted therapy is only successful in a limited number of patients. Since tyrosine kinase signaling is known to play important roles in diverse aspects of cancer biology [85], we hypothesize that also other tyrosine kinases are playing important roles in HNSCC biology which could be exploited for therapy. The important role of several other tyrosine kinases in HNSCC biology is indeed becoming apparent. [52,92,93].

Aims are:

1) Identify new tyrosine kinases that play an important role in cell survival and radioresistance in HNSCC.

2) Translate this knowledge to a clinically applicable form. When such a tyrosine kinase is identified we will try to exploit this knowledge as new targeting strategies (tyrosine kinase inhibitors, ligand sequestration, blocking antibodies) or as prognostic / predictive markers.

1.2 HPV in HNSCC: epidemiology and radiobiology

As explained in the introductory section HPV-related HNSCC is a distinct tumor entity forming a new epidemic. Because of its aberrant clinical behavior the question rises whether these tumors should be treated differently. To set up new studies, we need

to have an idea about the prevalence of HPV in oropharyngeal cancer in our patient population. These data are not available for Flanders.

Aims are:

1) Determine the presence of high-risk HPV in patients with oropharyngeal cancer in Flanders

2) Assess the impact of HPV in patients on treatment response and survival

3) Translational part: perform marker studies on biopsies of oropharyngeal cancer as well as functional studies on HPV positive versus negative HNSCC cell lines to better understand the biology of HPV- related HNSCC.

CHAPTER III

AN UNBIASED TYROSINE KINASE SCREEN IDENTIFIES KINASES WITH POSSIBLE IMPACT ON HEAD AND NECK SQUAMOUS CELL CARCINOMA SURVIVAL AND RADIORESISTANCE

Part of this work has been published as:

EJ. Van Limbergen, P. Zabrocki, M. Porcu, E. Hauben, J. Cools, S. Nuyts. FLT1 kinase is a mediator of radioresistance and survival in Head and Neck Squamous Cell Carcinoma. *Acta Oncologica, 2013; Early Online 1-9.*

1. Introduction: RNAi screen

Tyrosine kinases are, as discussed earlier in (chapter I.3) attractive targets in HNSCC. Although the role EGFR is well established in the radioresistance of this disease [36,44-46], the potential role of other members of the large TK's remains undetermined. Therefore we performed a high throughput screen of the whole tyrosine kinome to identify additional tyrosine kinases as novel targets to enhance the radiotherapy response in HNSCC. The basic setup of the screen was a dropout approach. Using shRNAmir vectors (short hairpin RNA vectors, based on microRNA backbone) we would 'knockdown' the expression a specific tyrosine kinase [146]. The assumption was that when the silenced tyrosine kinase was important for radioresistance, cells carrying an shRNAmir silencing such a kinase would die and disappear from the cell population after radiotherapy but not after general culturing. This would result in a dropout of the shRNAmir sequence from the cell cultures.

2. Materials and methods

2.1. Cell lines and culture conditions

The RNAi screen was performed on two human HNSCC cell lines derived from the larynx (SQD9) and tongue (SCC61) [147,148]. SQD9 and SCC61 were a gift from A.C. Begg (Netherlands Cancer Institute, The Netherlands). Cells were grown in DMEM containing 10% fetal bovine serum (FBS). HEK293T cells were used to generate lentiviral vectors. Cells were maintained at 37° Celsius in a humidified incubator with a 5% CO_2/95% air atmosphere.

2.2. Generation of lentiviral vectors

Lentiviral vectors were prepared using HEK279T cells and the Trans-Lentiviral shRNA Packaging System (Thermo scientific) according to the manufacturers protocol.

2.3. Primary lentivirus-based RNAi screen

Figure 3 shows an overview of the screen setup. The RNAi screen was conducted on the SCC61 and SQD9 HNSCC cell lines. A tyrosine kinase library based on 268 pGIPZ lentiviral shRNAmir vectors (Open Biosystems) was used. Every tyrosine kinase was targeted by multiple shRNAmir's. HNSCC cell lines were transduced to get stable cell lines with the expression of a single shRNAmir. Polybrene (8µg/ml) was added to increase transduction efficiency. Viral supernatant was removed after 24h. pGIPZ plasmids contained turboGFP allowing to monitor transduction efficiency using a FACSCanto flow cytometer (BD Bioscience). Cultures were split into three equal parts at day six. That day, one part was used to extract genomic DNA (gDNA) as a reference sample, a second part was irradiated with 4 Gy of ionizing radiation (Clinac, 6MV, dose rate 300cGy/min) and cultured, whereas the third part was cultured under standard conditions. Three weeks later gDNA was harvested from the cells under the experimental conditions. ShRNAmir specific sequences inserted in the gDNA were PCR amplified with generic primers. PCR reactions were monitored in order to obtain PCR products from the logarithmic phase of amplification. PCR products were sequenced using a GS-FLX 454 sequencer (Roche) according to manufacturer's prescriptions and then counted using CLC genomics workbench software (CLC bio). The relative appearance of each different viral vector in the cell population was determined and compared to the reference sample. ShRNAmirs representing less than 0.1% of the total shRNAmir count in the reference sample were excluded from further analysis. An shRNAmir was carried further into the screen as a potential candidate affecting radiosensitivity if the shRNAmir resulted in a cell loss of at least a factor 3, and if the cell loss was more pronounced in the irradiated condition as compared to the non-irradiated condition cultured in parallel. Further shRNAmir's that were completely lost in the non-irradiated experimental condition were carried through as kinases affecting survival. Primarily this was done because a potential additional radiosensitizing effect could not be evaluated with this screen setup as the shRNAmir already results in complete cell kill without radiotherapy. Additionally

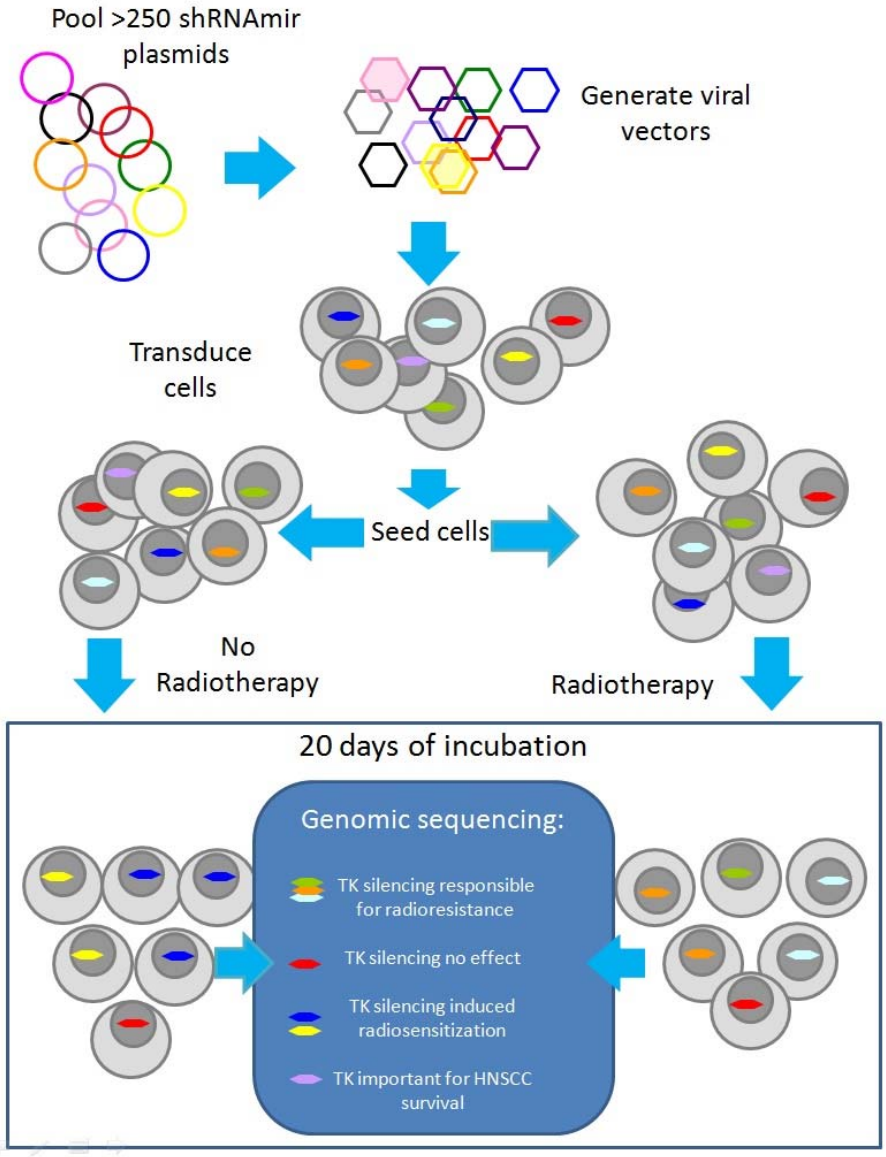


Figure 3: An overview of the primary lentivirus based RNAi screen. HNSCC cells were transduced with an shRNAmir library targeting the different members of the TK family. The transduced cell population was split in 2 parts, one receiving radiotherapy while the other part doesn't. GS-FLX sequencing of the shRNAmir-barcodes then allows to identify which tyrosine kinases are important for radioresistance of survival.

these hRNAmir's, most strongly affecting HNSCC cell survival could reveal interesting therapeutic targets for HNSCC cancer therapy as well. Hits from a first screening round were tested again in the same setup using smaller shRNAmir pools. Consistent hits were carried through to the follow-up screen setup.

2.4. ShRNA based follow-up screen

An overview of the setup of the follow-up screen is presented in figure 4. SQD9 and SCC61 cells were transduced with lentiviruses carrying individual shRNAmirs, leading to specific expression of turbo-GFP (Green Fluorescent protein) in transduced cells. GFP-positive transduced cells were mixed with GFP-negative non-transduced cells of the corresponding cell line in order to achieve cultures containing about 50% of GFP-positive cells. A non-silencing shRNAmir was used as the control condition. The percentage of GFP positive cells at a given timepoint ($P_{GFP\ (TP)}$) was determined by Fluorescence Assisted Cell Sorting (FACS) every 3-4 days. The GFP disappearing velocities (V_{GFP}) were calculated as $V_{GFP} = (P_{GFP\ (TP\ x)} - P_{GFP\ (TP\ x+1)})/\Delta t_{(TPx \rightarrow TPx+1)}$ and compared to the control condition using a one sided paired student's t-test. P-values of < 0.05 were considered significant. ShRNAmir's with a significant increase in V_{GFP} were considered as important for survival. To consider a hit as 'radiosensitizing' it was additionally required that the overall GFP decrease was more pronounced in the irradiated condition (1 x 4 Gy, Varian Clinac, 6MV, dose rate 300cGy/min) as compared to the condition without irradiation.

3. Results

The specific results of these screening experiments for each hairpin are provided in the addendum at the end of this chapter. Our screen set-up allowed us to identify *Fms*-Like Tyrosine kinase 1 (FLT1), also known as Vascular Endothelial Growth Factor Receptor 1 (VEGFR-1) as a tyrosine kinase affecting radiosensitivity as well as cell survival in SCC61 as well as SQD9. Further AnneX-eLectro receptor tyrosine kinase

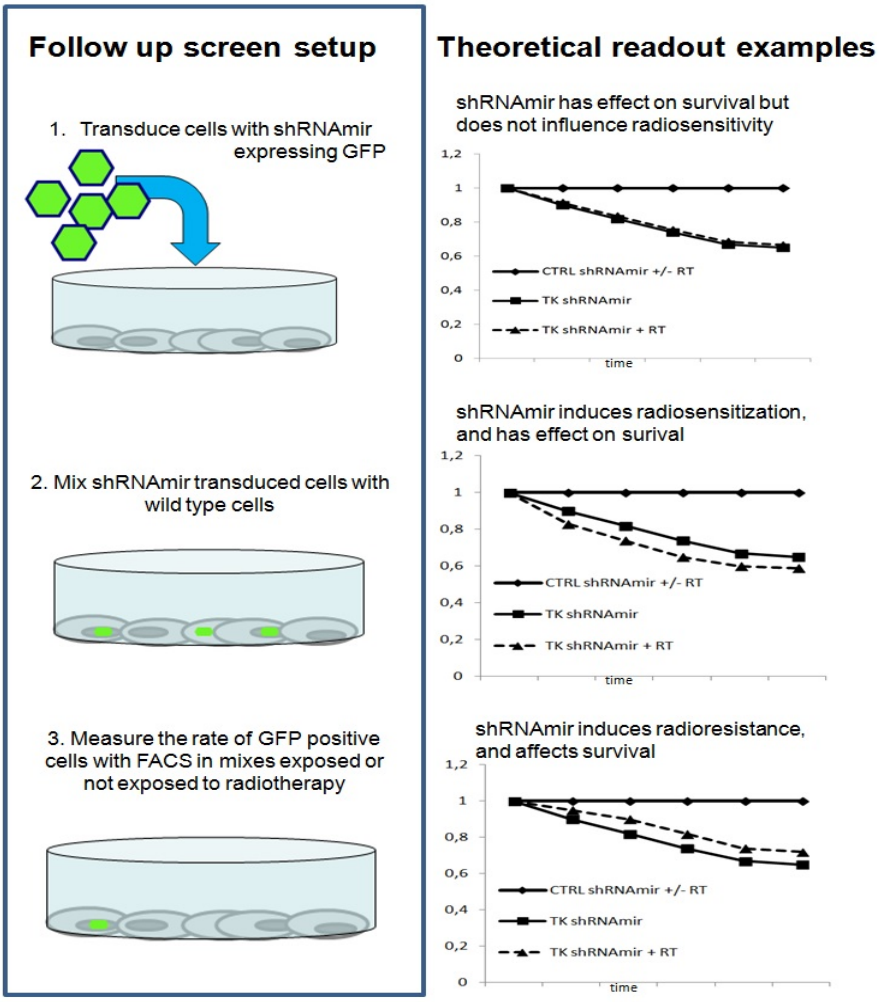


Figure 4: Left: Overview of the follow-up screen setup. Cancer cells were transduced with shRNAmirs containing a GFP expression vector.Transduced cells were then mixed with non-transduced cells at 50/50% rate. Half of the mixes were exposed to radiotherapy while the other half was only cultured in parallel. Rates of GFP positive cells were measured by FACS. Data were normalized to a non-silencing shRNAmir vector. GFP disappearing velocities were then compared to a non-silencing shRNAmir vector using a one sided TTEST. Right: theoretical readout examples of shRNAmirs affecting survival and/or radiosensitivity. To consider a hit as radiosensitizing it was additionally required that the overall GFP decrease was more pronounced in the irradiated condition

(AXL), Bone Marrow tyrosine kinase coded in chromosome X (BMX), and Tyrosine Non-receptor Kinase 1 (TNK1) were identified in SCC61 as potential tyrosine kinase affecting cell survival, whereas EGFR was suggested to affect radiosensitivity as well as cell survival. In SQD9 TXK tyrosine kinase (TXK) was identified as another tyrosine kinase potentially affecting cell survival, but not radiosensitivity.

4. Discussion

Our screen setup allowed for a more generalized and unbiased assessment of a possible role of the whole TK family in HNSCC cell lines. However we must note that this assessment may not have excluded a possible importance of every different member of the TK family to a complete extent. First of all, as can be seen from the data retrieved from our TK screen, not every TK was encompassed by the screen. ShRNAmirs targeting CSK, LMTK3 and MST1R or JAK2, JAK3 and PTK6 were never found after baseline sequencing just after transduction in SCC61 of SQD9 respectively meaning they were not evaluated. Reasons why an shRNAmir is not found may vary: it is possible that these vectors were not present in the viral pools, or that they for some reason fail to transduce a specific HNSCC cell line. Further, it is also possible that these vectors silence a highly essential TK for cell survival in this cell line. This would result in complete cell kill even before baseline sequencing, which is more problematic. Therefore this is a more important shortcoming of the screen-setup. However, since the baseline sequencing was done before exposure to ionizing radiation this shortcoming is less likely to interfere with the adequate evaluation of a possible role in radioresistance.

Further it is also possible that a shRNAmir vector is transduced successfully into a HNSCC cell but that the shRNAmir sequence fails to silence the expression of this specific TK. Then the screen readout would suggest that a TK with possible importance towards radiosensitivity would not be picked up as a dropout. This potential problem was partly countered by the fact that each member if the TK family

was targeted by multiple shRNAmirs in our experimental setup, hoping that at least one would be effective towards gene silencing.

Because shRNAmirs vary in their effectiveness in gene silencing, it is important to keep in mind that the relative importance of a specific kinase towards HNSCC radioresistance or survival can't be assessed directly from these screen data. It is indeed possible that a weaker dropout effect can be seen from poor knockdown of a highly essential TK, while a similar effect can be seen from perfect knockdown of a less essential TK.

Further from our own experience we report that sometimes shRNAmir vectors or siRNA's may induce cell kill in a way that is not related to the knockdown of a specific kinase, resulting in false positive hits.

Nonetheless this screen setup yielded several interesting candidates playing a potential role in HNSCC radiosensitivity or survival. Specifically FLT1, seeming of importance in HNSCC radiosensitivity as well as survival in both cell lines, was a finding of high interest. Extreme cautiousness was however still required interpreting these results (see higher described comments). Moreover, a possible role of FLT-1 in survival and radioresistance was currently only identified in two HNSCC cell lines. Therefore it also needed to be determined if FLT-1 is playing a role in a limited set of HNSCC or if it was of a more general importance. Further validation of the potential role of FLT 1 was therefore mandatory (see chapter IV).

ADDENDUM: shRNAmir screen result tables

CELL Line: SQD9

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR>non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
AATK\(199)	2,4748	1,1357	2,899	RR													
ABL1\(11)	0,2376	0,4788	0,7448	RR													
ABL1\(122)	0,0297	0	0,0721	N/P													
ABL1\(127)	0,6632	0,0111	0,3203	insuff													
ABL2\(118)	0,0495	0,1113	0	N/P													
ABL2\(200)	0,0594	0,9019	0,016	N/P													
ABL2\(206)	0	0	0,008	N/P													
ALK\(112)	0,8117	1,5811	1,3934	insuff													
ALK\(241)	0,0198	0,0334	0,048	N/P													
AXL\(102)	0,396	0,3452	0,8249	RR													
AXL\(128)	0	0	0,008	N/P													
AXL\(66)	0,9701	0,412	1,001	RR													
AXL\(73)	0	3,474	0,1121	N/P													
BLK\(133)	0	0	0,1441	N/P													
BLK\(163)	0,6632	1,3473	0,2563	insuff													
BLK\(223)	0,0594	0	0,008	N/P													
BLK\(62)	0,3168	0	0,0801	**Surv**	BLK\(62	0	0	0,0075	N/P	BLK\(62)							
BMX\(143)	0,8315	0,5011	0,04	**RS**	BMX\(14	0,3342	2,3154	4,7115	RR	BMX\(143)							
BMX\(158)	0,0297	0,4231	0,048	N/P													
BMX\(42)	0,6335	0,5567	0,3684	insuff													
BMX\(43)	0,6632	0,2227	0,4965	insuff													
BMX\(47)	0,0495	0	0	N/P													
BMX\(58)	1,3661	9,0636	3,5157	insuff													
BTK\(110)	0,0693	0,0334	0,008	N/P													
BTK\(204)	0,4158	0,1782	0,7688	RR													
CSF1R\(104)	0,0693	0,2116	0,1522	N/P													
CSF1R\(138)	0,5246	0,4899	0	**RS**	CSF1R\(	0,5013	2,7711	0	**RS**	CSF1R\(	0,4763	/	0,4391	/	/		
CSF1R\(149)	0,3762	0,5122	0	**RS**													
CSF1R\(22)	0,0693	0,1336	0,6086	N/P													
CSK\(72)	0	0	0,04	N/P													
DDR1\(117)	0,0198	0	0	N/P													
DDR1\(148)	0,7424	0	0	**Surv**	DDR1\(1	1,6908	0,5207	1,8861	RR	DDR1\(148)							
DDR1\(41)	0	0	0,008	N/P													
DDR1\(7)	0,3663	0	0	**Surv**													
DDR2\(51)	1,3166	0	0	**Surv**	DDR2\(5	1,4745	1,7761	0,0149	**RS**	DDR2\(5	0,2735	/	0,4633	/	/		
EGFR\(53)	0,1881	0,2004	0,008	**RS**	EGFR\(5	0,118	0	0	**RS?**	EGFR\(5	0,4611	/	0,3431	/	/		
EPHA1\(205)	0,8612	0,7126	1,5856	RR													
EPHA2\(13)	0	0,0223	0	N/P													
EPHA2\(224)	0,8909	0,2116	1,4815	RR													
EPHA3\(115)	1,0493	0,3786	2,3224	RR													
EPHA4\(106)	0,495	0,167	1,4255	RR													
EPHA4\(177)	0,0198	0	0	N/P													
EPHA4\(5)	0,0396	0,0891	0,2322	N/P													
EPHA4\(71)	0,2871	0,0111	0,0961	insuff													
EPHA5\(213)	0,1485	0,0334	0,1522	RR													
EPHA6\(203)	0,3861	0,4677	0,6006	RR													
EPHA6\(94)	0,0297	0,4231	0,4885	N/P													
EPHA7\(172)	0,0495	0	0	N/P													
EPHA7\(179)	0	0	0,7608	N/P													
EPHA8\(1)	0,2574	0	0	**Surv**	EPHA8\(	0,0098	0	0	N/P	EPHA8\(83)							
EPHA8\(154)	4,7119	2,2603	2,5707	insuff													
EPHA8\(83)	0,099	1,3807	0,1522	N/P													
EPHB1\(146)	0,3663	0,245	0,6246	RR													
EPHB1\(34)	0,584	0	0,2402	**Surv**													
EPHB1\(70)	0,2079	0	0,008	**Surv**	EPHB1\(	1,3074	0,5858	0,4547									
EPHB3\(130)	0,7721	0	0	**Surv**	EPHB3\(	0,4718	1,2089	0	**RS**	EPHB3\(	0,1657	/	0,4443	/	/		
EPHB4\(228)	0,3564	0,2338	0,2002	insuff													
EPHB4\(229)	0,0099	0,1448	0,008	N/P													
EPHB6\(132)	0	0,0557	0	N/P													
EPHB6\(140)	0,782	0,0891	0,0801	**RS**	EPHB6\(	1,4647	0,6137	2,4899	RR	EPHB6\(140)							
ERBB2\(234)	0,0099	0	0	N/P													
ERBB2\(92)	0,0297	0	0,016	N/P													
ERBB3\(182)	0,0099	0	0,7047	N/P													
ERBB3\(28)	0	0	0,2483	N/P													
ERBB4\(98)	0	0,5567	0,016	N/P													
ERBB4\(99)	0,2277	0,8351	0,4405	insuff													
FER\(131)	0,0792	0,0111	1,0971	N/P													
FER\(19)	0,0396	0	0	N/P													
FES\(119)	0,0099	0	0,0721	N/P													
FGFR1\(114)	0,0297	0	0	N/P													
FGFR2\(124)	0,6731	0	0,3924	**Surv**													
FGFR2\(145)	0,198	0	0,0721	**Surv**													
FGFR2\(152)	3,2964	1,9152	0,8649	**RS**	FGFR2\(	0,1671	1,5715	0,0895	Insuff	FGFR2\(124)							
FGFR2\(217)	0,1881	0	0	**Surv**													
FGFR2\(38)	0,3069	0,2672	0,1201	insuff													

CELL Line: SQD9

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR> non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
FGFR3\(150)	0,1287	0,0111	0,008	**RS**													
FGFR3\(153)	0,0693	0	0	N/P	FGFR3\	0,7176	0,6137	0	**Surv**	FGFR3	0,3243	/	0,1473	/	/		
FGFR3\(35)	4,0883	4,7545	2,8109	insuff													
FGFR3\(59)	0,0099	0	0,008	N/P													
FGFR\(21)	0,0099	0	0,008	N/P													
FLT1\(6)	0,3465	0	0	**Surv**	FLT1\(6)	0,2163	0	0	**Surv**	FLT1\(6)	**p<0,05**	**toxic**	**p<0,05**	**yes**	**yes**	**FLT1**	**RS & Surv**
FLT1\(89)	0,0297	0	0	N/P													
FLT3\(32)	0	1,7927	0,2803	N/P													
FLT3\(45)	0,5543	0,1336	0,5526	insuff													
FRK\(126)	1,6333	0,3452	1,0731	insuff													
FRK\(134)	0,8216	0,7126	1,0811	RR													
FRK\(142)	0,0099	1,1691	0	N/P													
FRK\(209)	13,72	10,511	24,578	RR													
FYN\(244)	0,0297	0	0	N/P													
FYN\(245)	0,1485	0	0	**Surv**	FYN\(24	0	0,0093	0	N/P	FYN\(245)							
HCK\(165)	0,0099	0,0111	0	N/P													
HCK\(192)	0	0	0,2402	N/P													
HCK\(218)	0	0	0,0961	N/P													
IGF1R\(14)	0,3762	0	0,5686	**Surv**													
IGF1R\(191)	0,297	0,2672	0,1281	insuff													
IGF1R\(57)	0,4158	2,7837	0,977	insuff	IGF1R\(5	9,4466	8,713	7,2089	insuff								
IGF1R\(60)	0,0396	0,0334	0	N/P	IGF1R\(6	0,3637	0,0372	1,5879	insuff								
IGF1R\(81)	0,0396	0,2784	0,1441	N/P													
ITK\(108)	2,2669	4,9549	1,4095	insuff													
ITK\(116)	0,2178	0	0,0881	**Surv**	ITK\(116)	2,9392	2,2131	1,3046	insuff								
ITK\(195)	0	0,0334	0,3444	N/P													
ITK\(25)	0,1287	0,1559	0,8649	RR													
ITK\(87)	0,1089	0,1336	0,5205	RR													
ITK\(96)	1,2869	0	1,5776	**Surv**													
JAK1\(211)	0,7919	0,1113	0,6487	insuff													
JAK1\(212)	0,9998	2,0822	0,4324	insuff													
JAK2\(170)	0,0297	0	0,032	N/P													
JAK2\(90)	0,2673	0,0111	0	**RS**	JAK2\(9	1,2484	0	0	**Surv**	JAK2\(9	0,1451	/	0,4813	/	/		
JAK3\(84)	0,1782	0	0,0641	**Surv**													
KDR\(147)	0,495	0,0334	0	**RS**	KDR\(14	0,6095	1,0229	0,1044	**RS**	KDR\(14	0,1125	/	**p<0,05**	**no**	/		
KDR\(222)	0,0099	0,0223	0,1281	N/P													
KDR\(54)	3,7715	3,3181	1,5136	insuff													
KDR\(95)	0,4059	0,0668	0	**RS**	KDR\(95	0,521	0,0186	1,1704	RR	KDR\(95)							
KIT\(121)	0,2475	0,7126	0,024	**RS**	KIT\(121)	1,7399	0,0837	3,966	RR	KIT\(121)							
KIT\(4)	0,2871	0,3674	1,3134	RR													
LCK\(240)	0	0,0111	0,3203	N/P													
LCK\(80)	1,0097	1,3473	0,7047	insuff													
LMTK2\(91)	0,1188	0	0	**Surv**													
LMTK3\(233)	0,4653	0,4565	0	**RS**	LMTK3\	0,0885	0	1,7295	N/P	LMTK3\(233)							
LTK\(15)	0,0594	1,4364	0,2322	N/P													
LYN\(166)	0	0,1336	0	N/P													
LYN\(202)	5,2168	5,5228	3,3955	insuff													
LYN\(79)	0,8315	0	1,2813	RR													
MET\(226)	0,0495	0,0111	0,5846	N/P	MET\(226)		0,0093		N/P	MET							
MET\(227)	0,2178	0	0,2002	**Surv**													
MST1R\(207)	0	0,0445	0,008	N/P													
MUSK\(17)	0,0099	0	0,1041	N/P													
MUSK\(208)	0,3366	0,0557	0,0961	RR													
NTRK1\(111)	0,0891	0	0,008	N/P													
NTRK2\(190)	0	0	0,2563	N/P													
NTRK2\(82)	0,8315	2,6834	2,2664	insuff													
NTRK2\(86)	1,1384	1,2137	0,0881	**RS**	NTRK2\	7,6182	13,446	8,5359	RR	NTRK2\(86)							
NTRK3\(137)	0,1881	0	0,008	**Surv**													
PDGFRA\(129)	0,5246	0,1893	0,2723	insuff													
PDGFRA\(139)	0,1089	1,1135	0,1522	N/P													
PDGFRA\(155)	2,158	1,1914	2,3945	RR													
PDGFRA\(183)	1,5541	1,2359	0,2483	**RS**	PDGFR	4,8363	4,3984	13,359	RR	PDGFRA\(183)							
PDGFRA\(194)	0,0495	0,2227	0	N/P													
PDGFRB\(225)	0,495	0,0223	0,04	RR													
PLK1\(242)	0,1881	0,1559	0,1201	insuff													
PTK2\(18)	0,1683	2,0488	0,008	**RS**													
PTK2\(188)	0	0	0,016	N/P													
PTK2\(214)	0,7424	0,412	1,4335	RR													
PTK2\(52)	0,2574	0,7906	0,048	**RS**	PTK2\(5	0,9437	2,4735	0,0373	**RS**	PTK2\(5	0,0756	/	0,1809	/	/		
PTK2\(77)	0,3069	0	0,008	**Surv**													
PTK7\(156)	0,1287	0,0111	0,5766	RR													
RBX1\(237)	0,2574	0,9464	0,1041	insuff													
RBX1\(238)	0,5939	0,0111	0,016	RR													
RBX1\(239)	0	0,0111	0	N/P													
RET\(101)	0,0099	0	0	N/P													

CELL Line: SQD9

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR>non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
RORl\(144)	0,5741	0,4008	0,1361	**RS**	RORl\(1	3,1063	0,5579	4,8233	RR	RORl\(144)							
ROR2\(197)	0,0099	0,0223	0	N/P													
ROS\(125)	0,881	0,1113	0,7528	RR													
RYK\(136)	0,0198	0,9019	0,3444	N/P													
RYK\(67)	0	0,0557	0,0561	N/P													
SRC\(246)	0	0,0111	0	N/P													
SRC\(44)	1,475	0,3786	1,2893	RR													
SRMS\(210)	0,0297	0	0,016	N/P													
STYK1\(109)	0	0,0111	0,008	N/P													
STYK1\(157)	1,3166	1,4141	0,5366	insuff													
SYK\(123)	0,4356	0	0,008	**Surv**													
SYK\(189)	2,4748	0,4343	0,048	**RS**	SYK\(189	3,5388	1,8226	5,6583	RR	SYK\(189)							
SYK\(88)	0,1782	0,1559	0,024	**RS**	SYK\(88	0,4227	0,3069	5,8521	RR	SYK\(88)							
TEC\(141)	3,8012	0,6235	1,0571	RR													
TIE2\(23)	0,0099	0	0	N/P													
TIE2\(33)	0,1584	0,1225	0,2723	RR													
TIE2\(8)	0,6731	0,0445	0,1842	RR													
TNK1\(50)	0,0198	0,4677	0,016	N/P													
TNK1\(61)	0,0099	0,0445	0,0721	N/P													
TXK\(159)	0,6632	0,0779	0,1762	RR	TXK\(159	1,3074	0	0	**Surv**	TXK\(159	**p<0,05**	**toxic**	0,3157	/	/	**TXK**	**Surv**
TXK\(160)	0,2772	1,0133	1,3454	RR													
TXK\(46)	0,7325	0,8462	1,3774	RR													
TXK\(68)	0,4158	0,3786	1,1452	RR													
TXK\(78)	0,1584	0	0	**Surv**													
TYK2\(63)	0	0	0,2483	N/P													
YES\(201)	1,0394	1,6368	2,3705	RR													
YES\(85)	0,0099	0	0,3363	N/P													
ZAP70\(113)	0,1584	0,2784	0	**RS**	ZAP70\(	3,0473	0,3813	0	**RS**	ZAP70\(	0,44	/	0,4356	/	/		
ZAP70\(135)	0	0,0111	0	N/P													
ZAP70\(196)	0	0	0,4965	N/P													

Legend: RS, radiosensitizing; RR, inducing radioresistance; N/P, not present; Surv, survival

CELL Line: SCC61

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR> non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
AATK\(199)	1,151	0,9198	0,9468	insuff													
ABL1\(11)	0,0311	4,9055	0,112	N/P													
ABL1\(122)	0	0,0083	0	N/P													
ABL1\(127)	1,1717	0	0,0305	**Surv**	ABL1\(12	1,3868	3,2606	4,4487	RR	ABL1\(127)							
ABL2\(200)		0,7292	0	N/P													
ALK\(112)	0,0104	2,4776	1,1707	N/P													
ALK\(241)	0,3422	0,0166	0,0102	**RS**	ALK\(24	0,0185	0,7821	0,0073	N/P	ALK\(241)							
AXL\(102)	0	2,5688	0,1629	N/P													
AXL\(128)	0	0	0,0102	N/P													
AXL\(66)	3,9818	0,4226	0,0509	**RS**	AXL\(66	1,9138	0	0,0073	surv	AXL\(66	**p < 0,05**	**toxic**	0,327242	/	/	**AXL\(66)**	**Surv**
AXL\(73)	0,1244	0,0746	0	**RS**													
BLK\(133)	1,4932	0,0249	0,5803	insuff													
BLK\(163)	0,0622	0,4475	0	N/P													
BLK\(223)	0,1452	0	0	**Surv**	BLK\(133	0	0	0,0073	N/P	BLK\(133)							
BLK\(62)	0	2,4528	0,2545	N/P													
BMX\(143)	0,4562	0	0,0611	**Surv**	BMX\(14	4,4656	0	1,5096	surv	BMX\(14	**p < 0,05**	**toxic**	0,054401	/	/	**BMX\(143**	**Surv**
BMX\(158)	0,0933	0	0,0102	N/P													
BMX\(42)	0,4251	0	0,0102	**Surv**													
BMX\(43)	0,2281	0	0,5497	**Surv**													
BMX\(47)	0,0104	0	0	N/P													
BMX\(58)	0,3111	0,2237	0,9569	RR													
BTK\(110)	0,0622	0	0	N/P													
BTK\(204)	6,2215	0,1906	0,1018	**RS**	BTK\(20	4,4101	8,46	6,7824	RR	BTK\(204)							
CSF1R\(104)	0,0933	0,2154	0	N/P													
CSF1R\(138)	0	0	0,0102	N/P													
CSF1R\(22)	0	0	0,0102	N/P													
DDR1\(117)	0	0	0,2545	N/P													
DDR1\(148)	0,197	0	0	**Surv**	DDR1\(1	0,2311	0,033	2,888	RR	DDR1\(148)							
DDR1\(41)	0,0104	0	0,0713	N/P													
DDR1\(7)	0,0207	0	1,242	N/P													
DDR2\(51)	0,0104	0,1823	0	N/P													
EGFR\(53)	0,5392	0	0,7228	**Surv**	EGFR\(5	0,0092	0	2,8369	**Surv**	EGFR\(5	**p < 0,05**	**toxic**	**p < 0,05**	**toxic**	**yes**	**EGFR**	**RS & Surv**
EPHA1\(205)	0,083	0,1326	0,5497	N/P													
EPHA2\(224)	0,1141	0,0249	0	**RS**	EPHA2\(	6,3332	20,346	11,647	RR	EPHA2\(224)							
EPHA3\(115)	1,462	2,7014	6,5866	RR													
EPHA4\(106)	1,265	0,0249	3,0642	RR													
EPHA4\(5)	0,0518	0	0,0102	N/P													
EPHA4\(71)	0,0104	0,0331	0,1629	N/P													
EPHA5\(213)	0,2281	0	0,0102	**Surv**	EPHA5\(	1,128	4,8249	5,8197	RR	EPHA5\(213)							
EPHA6\(203)	0	1,1269	0,0204	N/P													
EPHA6\(94)	0,0622	0,0663	0,2341	N/P													
EPHA7\(172)	0	3,5549	0	N/P													
EPHA8\(154)	3,7847	2,5025	7,7878	RR													
EPHA8\(83)	0,3111	0,0083	4,1841	RR													
EPHB1\(146)	0,0415	0,6795	0	N/P													
EPHB1\(70)	0,0622	0	1,8426	N/P													
EPHB3\(130)	0	0,0083	0	N/P													
EPHB4\(228)	1,0265	0,1243	0,0102	**RS**	EPHB4\(	0	0	0	N/P	EPHB4\(230)							
EPHB4\(229)	0	0	0,0204	N/P	EPHB4\(	0	0,011	0	N/P	EPHB4\(229)							
EPHB6\(140)	0	0	0,5396	N/P													
ERBB2\(234)	0	0,0083	0,0102	N/P													
ERBB2\(92)	0,0104	0	0,1323	N/P													
ERBB3\(28)	1,1925	0	0	**Surv**	ERBB3\(	0,037	0,0661	0	N/P	ERBB3\(28)							
ERBB4\(98)	0,1659	0	0	**surv**													
ERBB4\(99)	0,5496	1,1684	0,0204	**RS**	ERBB4\(	0,2866	1,8176	1,4367	RR	ERBB4\(99)							
FER\(131)	1,6383	0	0	**surv**	FER\(13	6,2962	3,1174	1,6932	**RS**	FER\(13	0,11591	/	0,433407	/	/		
FES\(119)	1,1199	0	0	**Surv**	FES\(119	0,4808	0,011	0	**RS**	FES\(119	0,05454	/	0,053536	/	/		
FGFR1\(114)	0	0	1,975	N/P													
FGFR1\(181)	0	0,0083		N/P													
FGFR2\(124)	0	0,0083	0,0204	N/P													
FGFR2\(145)	1,5243	0	0	**Surv**	FGFR2\(	0	0	0	N/P	FGFR2\(145)							
FGFR2\(152)	1,5865	0,5966	0,0407	**RS**													
FGFR2\(217)	0,0207	0	0	**N/P**													
FGFR2\(38)	0,6947	0	0,3461	**Surv**													
FGFR3\(153)	0,0311	0	0	N/P													
FGFR3\(35)	7,0199	4,831	5,1614	insuff													
FGFR3\(59)	0	0,0249	0,0102	N/P													
FGR\(21)	0	0,1077	0	N/P													
FLT1\(6)	0,3318	0,0083	0	**RS**	FLT1\(6)	4,632	0	0,1604	**Surv**	FLT1\(6)	**p < 0,05**	**toxic**	**p < 0,05**	**toxic**	**yes**	**FLT1**	**RS & Surv**
FLT1\(89)	1,7005	0	0	**Surv**													
FLT3\(32)	0	0	0,0102	N/P													
FLT3\(45)	0	1,5247	0,0102	N/P													

CELL Line: SCC61

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR> non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
FRK\(126)	0,0104	0	5,2632	NP													
FRK\(134)	0	0,0083	0	NP													
FRK\(142)	0,0104	0,0083	0	NP													
FRK\(209)	12,92	25,605	5,3446	insuff													
FYN\(245)	0	5,2287	0	NP													
HCK\(165)	0,0104	0	0	NP													
HCK\(165)	0	0,0166	0	NP													
IGF1R\(14)	0,3526	0	0	**surv**													
IGF1R\(191)	0	0	0,0102	NP													
IGF1R\(57)	0,0518	0,4972	2,5552	NP													
IGF1R\(60)	0	0	0,112	NP													
IGF1R\(81)	2,499	0,0166	0,4072	RR	IGF1R\(8	3,3561	0,2974	0,6491	RR	IGF1R\(81)							
ITK\(108)	2,0842	3,2731	4,1942	RR													
ITK\(116)	0,197	0	0,1527	**Surv**													
ITK\(195)	0,0104	0	0,5497	NP													
ITK\(25)	0,7881	0,6215	0,0916	**RS**													
ITK\(87)	0,4251	0,1657	0	**RS**	ITK\(87)	3,5503	0,2203	5,5134	RR	ITK\(87)							
ITK\(96)	0,0622	0,0414	0	NP													
JAK1\(211)	3,3492	0,5386	1,9851	insuff													
JAK1\(212)	0,083	0,0994	0,5599	NP													
JAK2\(170)	0	0,0912	0,7024	NP													
JAK3\(64)	0,0104	0,9861	0	NP													
KDR\(147)	0,0622	0	0	NP													
KDR\(54)	1,6902	2,9914	6,5051	RR													
KDR\(95)	0,6636	0,1243	1,8528	RR													
KIT\(121)	0,7466	0,0083	0,4479	insuff													
KIT\(4)	0,0311	0	0,2138	NP													
LCK\(80)	0	0,0083	0	NP													
LMTK3\(232)	0,1244	0	0	survival													
LMTK3\(233)	1,2443	0	0	survival	LMTK3\	0	0,4516	0	NP	LMTK3\(233)							
LTK\(15)	0	0,0663	0	NP													
LTK\(74)	0,0311	0	0	NP													
LYN\(202)	7,2999	3,5217	4,8356	RR													
LYN\(79)	0	0	0,2647	NP													
MET\(226)	0	0,0083	0	NP													
MET\(227)	0,0104	0	0	NP													
MUSK\(17)	0	0,9695	0	NP													
MUSK\(208)	0,2696	0	0	**Surv**	MUSK\(	0,2681	1,7845	4,5872	RR	MUSK\(208)							
NTRK1\(111)	0,0104	0	0,6719	NP													
NTRK2\(190)	0,7777	0,4392	0	**RS**													
NTRK2\(82)	0,4459	1,301	0,0407	**RS**	NTRK2\	2,7644	13,814	5,7395	RR	NTRK2\(82)							
NTRK2\(86)	0,9747	2,2788	4,0619	RR													
NTRK3\(137)	0	0	0,1222	NP													
PDGFRA\(129)	0,083	0,1409	0	NP													
PDGFRA\(139)	2,0738	0	0,0102	**Surv**	PDGFR	0,6102	0	0	**surv**	PDGFR	0,057195	/	0,23787	/	/		
PDGFRA\(155)	0,3007	2,2622	5,1308	RR													
PDGFRA\(183)	0,0518	0,5055	0,9977	NP													
PDGFRA\(194)	0,1555	0	0,1018	**Surv**													
PDGFRB\(225)	0,0207	0,6463	0	NP													
PTK2\(18)	0	0,3232	0,0204	NP													
PTK2\(214)	0,5185	0,3729	0,2341	insuff													
PTK2\(77)	0,6844	0	0,1323	**Surv**													
PTK6\(216)	0	0	0,0102	NP													
PTK7\(156)	0,0207	0	0,1323	NP													
RET\(101)	0	0	0,112	NP													
ROR1\(144)	1,9909	0,0083	1,1096	insuff													
ROR2\(197)	3,9299	0,0166	0	**RS**	ROR2\(	0	0,033	0	NP	ROR2\(197)							
ROS\(125)	0,4148	0,4392	0	RS	ROS\(12	9,375	4,0758	5,7978	insuff	ROS\(125)							
RYK\(136)	0,083	0,0083	0,0102	NP													
RYK\(67)	0,3318	0,0083	0,4377	RR													
SRC\(44)	0,0622	1,0358	0,6719	NP													
SRMS\(210)	0,7673	0,0083	0	**RS**	SRMS\(	0,1202	3,1835	1,2033	RR	SRMS\(210)							
STYK1\(109)	0,0933	0,0083	0,0102	NP													
STYK1\(157)	0,0311	0	0	NP													
SYK\(123)	0,0622	0	0	NP													
SYK\(189)	0	0,5386	0	NP													
SYK\(88)	0	0,0083	0	NP													
TEC\(141)	2,7582	0,6381	3,1457	RR													
TIE2\(219)	0,394	0,3397	1,4761	RR													
TIE2\(23)	0,0311	0	0,4276	NP													
TIE2\(33)	0,0726	0,7458	0,8144	NP													
TIE2\(8)	0	3,8615	0,0102	NP													

CELL Line: SCC61

initial screen											Follow-up screen						
first screen					repeat						Non-IRR mixes		IRR mixes				
Tyrosine kinase	measured abundance			Reason of exclusion		measured abundance			hits		p-value	shRNA more toxic	p-value	shRNA more toxic	toxicity IRR> non-IRR	tyrosine kinase hit	
	init	contr	irr			init	contr	irr									
TNK1\(61)	0,7362	0	0	**Surv**	TNK1\(6	4,7892	2,115	0,2698	**RS**	TNK1\(6	**p < 0,0**	**toxic**	**p < 0,05**	**yes**	not consistently	**TNK1(61)**	**Surv**
TXK\(160)	0,1763	0,1077	0,7126	RR													
TXK\(46)	0,2178	0,8866	0,8246	RR													
TXK\(68)	0,0104	2,2622	0	N/P													
TXK\(78)	0,0104	0	0,7635	N/P													
TYK2\(63)	0,0104	0	0	N/P													
YES\(201)	2,1983	0,1823	0,2036	RR													
YES\(76)	0,0104	0,0083	0	N/P													
YES\(85)	0	0,0414	0	N/P													
ZAP70\(113)	0,0104	0,0083	0	N/P													
ZAP70\(135)	0,5357	0,4945	0,5921	RR													
ZAP70\(196)	0,5346	0,4929	0,5917	RR													

Legend: RS, radiosensitizing; RR, inducing radioresistance; N/P, not present; Surv, survival

CHAPTER IV

FLT1 MEDIATES RADIORESISTANCE AND SURVIVAL IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS, THROUGH AUTOCRINE PRODUCTION OF VEGFA

Part of this work has been published as:

EJ. Van Limbergen, P. Zabrocki, M. Porcu, E. Hauben, J. Cools, S. Nuyts. FLT1 kinase is a mediator of radioresistance and survival in Head and Neck Squamous Cell Carcinoma. *Acta Oncologica, 2013; Early Online 1-9.*

1. Introduction

Previously FLT1 was identified as a possible target to modify survival and radiosensitivity in our HNSCC cell lines.

FLT1 forms together with FLK (VEGFR2) and FLT4 (VEGFR3) the VEGF-Receptor Tyrosine Kinase family. These receptors consist of seven extracellular immunoglobulin like domains, a trans-membrane region and a tyrosine kinase domain [149]. FLT1 binds VEGFA, VEGFB and PlGF, whereas FLK binds VEGFA and VEGFC. The role of FLK (and VEGFA) as the primary driving force of angiogenesis is well established. The role of FLT1 in this context is more elusive. The current belief is that FLT1 primarily acts as a decoy receptor, reducing FLK activity by sequestering stimulatory VEGFA. Consistent with this model, FLT1 has been shown to bind VEGFA with much higher affinity than FLK, although showing much less tyrosine kinase activity [149,150]. In accordance, loss of FLT1 expression causes embryonic lethality as a result of vascular overgrowth, while FLT1 kinase dead variants with normal VEGFA binding capacity do show normal vascular development and good health [151,152].

Other evidence, however, indicates that the role of FLT1 in angiogenesis is more complex, as several reports have demonstrated that FLT1 mediated intracellular signalling regulates angiogenesis in several pathological conditions, one of which is cancer [150]. In this context, numerous authors have shown that FLT1 inhibition suppresses tumour growth and metastasis [150].

More recent reports also demonstrate FLT1 expression directly on tumour cells from breast, colon and skin origin, and show it to be an important oncogenic driver in these cells promoting survival, cell proliferation, invasiveness and/or motility, in an angiogenesis-independent manner [153-156].

In this chapter we examine and validate the role of FLT1 radioresistance and survival in HNSCC .

2. Materials and Methods

2.1. Cell lines

Five HNSCC cell lines were used: SQD9, SCC61, SC263, Cal27, SCC154 and SCC090. Cells were grown in DMEM containing 10% fetal bovine serum (FBS). SCC154 and SCC090 cells were cultured in MEM containing 10 % FBS, 1% MEM Non Essential Amino Acids and 1% L-glutamine. Cells were maintained at 37° Celsius in a humidified incubator with a 5% CO_2/95% air atmosphere.

2.2. *SiRNA transfection*

Transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturers prescriptions. Non-silencing control and FLT1 stealth select siRNA's (short interfering RNA's) were obtained from Invitrogen (12935-400, HSS103744 and HSS103745, designated as control siRNA, FLT1 siRNA1 and FLT1 siRNA2 respectively).

2.3. Western blotting

Cells were lysed on ice with chilled cell lysis buffer (Cell Signaling) with additional Complete protease inhibitor (Roche) and sodium orthovanadate (6 mM final concentration). Proteins were separated on 4-12% NUPAGE gels (Invitrogen) and were blotted onto PVDF (PolyVinyliDene Fluoride)-membranes (Biorad). Membranes were blocked with 5% (w/v) non fatty milk in 1X TBS (Tris-Buffered Saline solution) containing 0.2% Tween-20. Following primary antibodies were used:, anti-□-Actin (#4967) anti-FLT1 (ab2350) (Abcam) and anti-phospho-tyrosine (4g10) (Millipore). HRP (Horse Radish Peroxidase)-linked secondary antibodies were used. Detections were performed with an ECL (Electrogenerated ChemiLuminescence) detection system (Perkin Elmer) using an ECL imager (LAS-3000 mini). For FLT1 immunoprecipitation the FLT1 antibody (ab2350) (Abcam) was covalently coupled to Dynabeads (Invitrogen) according to the manufacturers protocol.

2.4. Real-Time-qPCR and sequencing

Messenger RNA was extracted from cells using the RNeasy mini Kit (Qiagen) with additional on column DNA digestion. For siRNA knockdown analysis, mRNA was harvested 48h after transfection. Experiments were carried out on a Lightcycler 480 II (Roche). Primer sequences are listed in Table 1. The PCR cycle conditions were as follows: a preincubation step for 10 minutes at 95°C followed by 40 amplification cycles; each cycle included 10 seconds at 95°C and 30 seconds at 60°C; followed by melting curve analysis to verify specific amplification of the amplified product.

FLT1 sequencing was performed at the VIB service facility in Antwerp (Belgium).

TABLE 1: REAL TIME-qPCR PRIMER sequences

Gene	Primer sequence
FLT1 *	**F:** 5'- TTGCTGTGGGAAATCTTCTCCTTA -3'
	R: 5'- TGCCTTCCCTCAGGCGA -3'
VEGFA	**F:** 5'- CCATGCAGATTATGCGGATCA -3'
	R: 5'- GCATTCACATTTGTTGTGCTGTA -3'
VEGFB	**F**: 5'- TCCCTGAGGCCATCATCAA -3'
	R: 5'- CTGAGCTGGTATGTGACCC -3'
PlGF	**F:** 5'- CGTCGTGTCCGAGTACC -3'
	R: 5'- AGTGCAGATTCTCATCGCC -3'
HPRT	**F:** 5'- TGACACTGGCAAAACAATGCA -3'
	R: 5'- GGTCCTTTTCACCAGCAAGCT -3'

**, The primer set of FLT1 was taken over exons 24-25, thereby omitting possible detection of the FLT1 splice variants without tyrosine kinase domain [159].*

2.5. Sulphorodamine B (SRB) Survival assays

Cells were seeded one day before treatment. Cells were trypsinized and replated in 96-well plates 48h after transfection and then grown for another 72h before analysis. Non-silencing siRNA was used for the control conditions. Readout was performed using an SRB-assay, as previously described [157]. In short, cells were fixed after treatment with trichloric acid and stained with sulphorodamine B (SRB). The incorporated SRB was liberated from cells in a TRIS-base solution, and optical densities at 570 nm were determined. Cell survival was determined as the relative absorption of SRB in treated wells in comparison to controls.

2.6. Clonogenic assays

Experiments were carried out as previously described [157]. Radiotherapy was performed 72h after transfection and non-silencing siRNA was used in the control conditions. The plating efficiency (PE) was defined as the ratio of cells forming a colony of more than 50 cells over the amount of seeded cells after an incubation period of 2-3 weeks receiving no radiation. The survival fraction was calculated after normalizing the amount of colonies formed after having received a certain dose of radiotherapy to the plating efficiency. The dose enhancement factor was calculated as the ratio of the irradiation dose needed in the control condition over the irradiation dose needed in the experimental condition to obtain a survival fraction of 0.5.

2.7. Patient samples, immunohistochemistry (IHC) and survival analysis.

Formalin fixed paraffin embedded (FFPE) tumor samples were obtained from patients diagnosed with squamous cell carcinoma of the head and neck between 2000 and 2010 (larynx 10 samples, and oropharynx 139 samples). Four µm sections were dewaxed, rehydrated and peroxidase blocked according to standard protocols, followed by an antigen unmasking procedure using citrate buffer (FLT1-VEGFB) or DAKO high pH target retrieval solution (VEGFA). To minimize aspecific immunoreactivity, a blocking step was performed with protein block solution (X0909,

DAKO). The following primary antibodies were used: anti-FLT1 (1:200; Atlas antibodies, HPA011740); anti-VEGFA (1:100; SC-152, Santa Cruz); anti-VEGFB (1:20; MAB751, R&D systems). Secondary antibody detection was performed with biotinylated goat anti-polyvalent antibody together with HRP-linked streptavidin (Thermo Scientific) for FLT1 and VEGFB. Envision (DAKO) was used for VEGFA. Diaminobenzidine complex (DAB) was used as chromogen. Primary antibodies were omitted in the negative controls. Counterstaining was performed with hematoxylin.

The specificity of the FLT1 antibody HPA011740 on formalin fixed material was confirmed by immunocytochemistry on FLT1 siRNA treated SQD9 cells (see further).

FLT1 samples were scored by expression pattern (absent expression, expression within the basal layers or expression within the full epithelial thickness), or, alternatively, by staining intensity (absent, mild or strong staining intensity). Initially FLT1 staining was performed on 12 samples retrieved from surgically resected squamous cell carcinoma specimen of the larynx. In a second phase FLT1 staining was performed on an additional 139 biopsies of patients diagnosed with squamous cell carcinoma of the oropharynx. Of these 139 samples stained in total, 93 patients received curative doses of (chemo)radiotherapy ($\geq$ 60Gy) on macroscopic tumor, and were selected for survival analysis. The mean follow-up for this group was 618 days. Survival analysis was performed according to the Kaplan Meier method using STATISTICA11 software.

3. Results

3.1. FLT1 is expressed in HNSCC cell lines and its knockdown decreases cell survival and sensitizes for the effects of ionizing radiation

Since FLT1 affected both cell lines, we decided to focus on this kinase. In a first step of validating the role of FLT1, we used real time quantitative PCR (qPCR) and western blot to confirm FLT1 expression (Fig. 5A-B). We next determined if siRNA mediated FLT1 knock-down could mimic the effects observed with shRNAmirs. Using two

different siRNAs we confirmed decreased survival upon FLT1 knockdown. Survival decreased in both cell lines to 52% and 71% in SQD9 and 72% and 72% in SCC61 for FLT1 siRNA1 and 2 respectively (Fig. 5A-D). The radiosensitizing potential of FLT1 was also investigated in a clonogenic assay using different siRNAs targeting FLT1 in both cell lines. FLT1 silencing affected Plating Efficiency (PE). The averaged PE's for non-irradiated siRNA treated cells were as follows: 0.12, 0.09 and 0.06 in SCC61 and 0.16, 0.10 and 0.13 in SQD9 for control siRNA, FLT1 siRNA1 and FLT1 siRNA2 respectively. To rule out the possibility that the decreased cell survival seen in the colony assays after FLT1 silencing could be attributable only to direct cytotoxicity of the FLT1 siRNA (and not to a synergistic interaction with radiation), we generated the survival curves after normalizing to the PE for each siRNA. In this way differences in non-radiotherapy associated toxicity between the different siRNA's were equalized and therefore a difference seen in the survival curves should indicate radiosensitization. FLT1 silencing did affect radiosensitivity in both HNSCC cell lines (Fig. 5E-F) with the dose enhancement factor of the best working FLT1 siRNA being 1.62 and 1.20 in SQD9 and SCC61 respectively.

3.2. FLT1 is overexpressed in primary head and neck carcinoma

In order to confirm expression of FLT1 in patient samples we first validated the specificity of our FLT1 antibody on formalin fixed HNSCC cells (Fig. 6A-C). Initially, we investigated twelve formalin fixed paraffin embedded HNSCC tumor samples by immunohistochemistry (IHC) for expression of FLT1 and ligands (see further). All samples showed FLT1 expression. To confirm these findings, FLT1 stainings were performed on 139 additional samples, finding FLT1 expression in 124 (89%) of these samples. In the whole group of 151 samples 85 sections also contained normal epithelium. To compare FLT1 expression between tumor and normal epithelium, a semi-quantitative scoring system was used: absence of expression (score I), FLT1 expression in the basal layers (score II) and FLT1 expression in the full epithelial thickness (score III) (Fig. 6D-E). FLT1 expression was significantly upregulated in

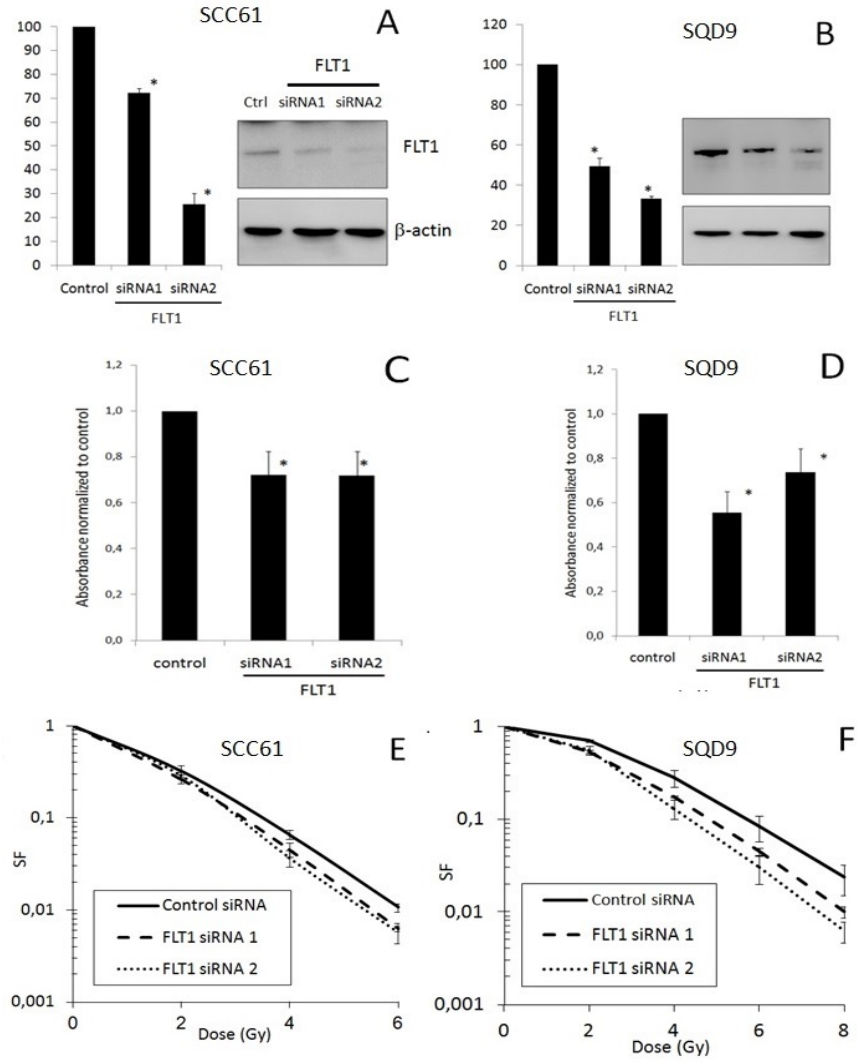


Figure 5: ***FLT1 is important for cell survival and radioresistance.*** *(A,B) SiRNA mediated knockdown of FLT1 was confirmed by qPCR (left) and western blot (right). (C, D) SRB survival assays performed with siRNA transfected SCC61 (C) and SQD9 (D) cells. (E,F) FLT1 silencing causes radiosensitization in SCC61 and SQD9 as demonstrated by clonogenic assay. Data shown are the averaged means of at least three independent experiments ± SEM and compared to non-silencing control siRNA treated cells. *, significantly different from control siRNA, p<0.05, students t-test. SF, survival fraction*.

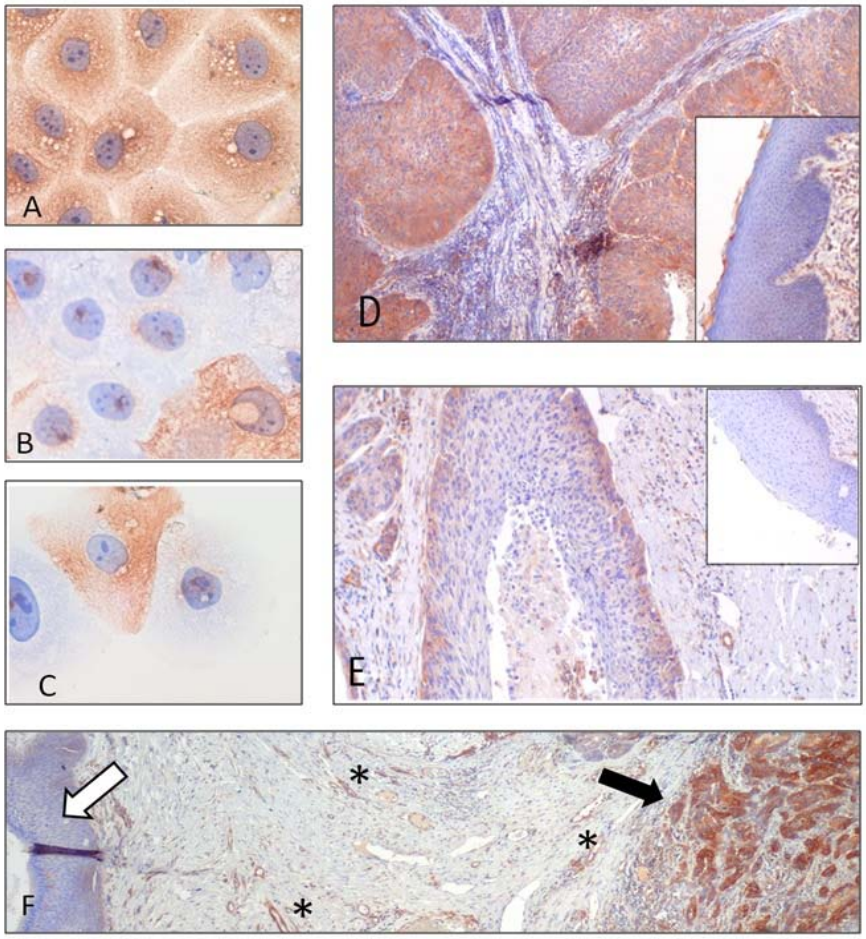


Figure 6: ***FLT1 is overexpressed in human HNSCC.*** *(A-C) The specificity of the FLT1 staining (shown in brown) was examined by immunocytology of formalin fixed FLT1 silenced SQD9 cells: (A) non-silencing control treated cells (400x); (B-C) FLT1 silenced cells. FLT1 positive cells in the FLT1 silenced conditions are expected since siRNA delivery is not equally effective in all cells. These positive cells prove that the FLT1 staining was performed correctly. (400x) (D-F) Immunohistochemistry on resected HNSCC. (D-E) show images of HNSCC samples with FLT1 expression (brown) in the full epithelial thickness, or expression limited to the basal layers respectively (40x). Corresponding normal epithelia are shown as insets (x100). (F) Immunohistochemistry of a HNSCC resection specimen showing FLT1 expression in the tumor (black arrow) but none in the normal epithelium (white arrow). Blood vessels used as an internal positive control for FLT1 staining are marked by '*' (40x).*

tumor compared to normal epithelia ($p<0.05$, Wilcoxon matched pairs test) as in tumor 15 (10%), 40 (26%) and 96 (64%) of samples were classified as score I, II and III, respectively, while in normal tissue 33 (39%), 49 (58%) and 3 (3%) samples received score I, II and III (Fig. 6E-F). FLT1 staining pattern nor FLT1 staining intensity in patient samples was predictive for locoregional control or overall survival (Fig. 7).

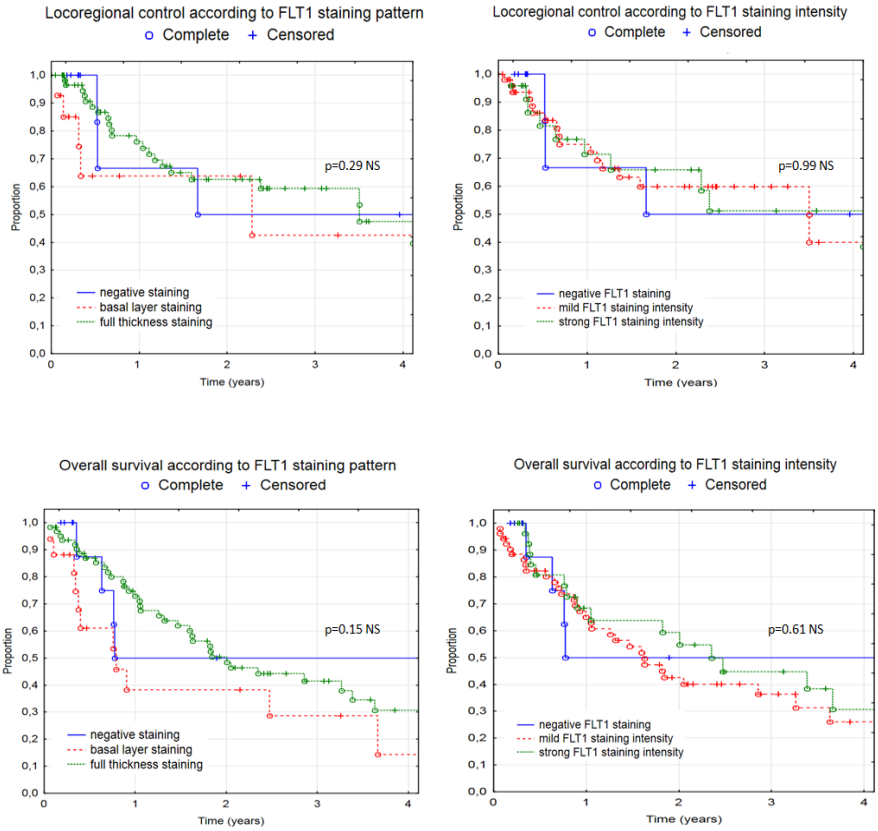


Figure 7: ***Locoregional control and overall survival in patients with oropharyngeal squamous cell carcinoma of the head and neck****, treated with radiotherapy, according to immunohistochemical FLT1 staining pattern or staining intensity. Data are presented as Kaplan-Meier survival curves.*

3.3. FLT1 ligands are produced in HNSCC cell lines and primary tumours and correlate with radioresistance

FLT1 immunoprecipitation experiments documented phosphorylation of the receptor in both HNSCC cell lines used in the screen, suggesting activation of this receptor in these cells (Fig. 8A *inset*). After sequencing exons 2-30 of the FLT1 receptor in SQD9 and SCC61 we found no evidence of activating mutations. We then determined expression levels of FLT1 ligands which could result in receptor activation [150]. We documented a strong expression of VEGFA and VEGFB in SQD9 and SCC61. PlGF expression was negligible (Fig. 8A).

Interestingly, VEGFA, having a FLT1 binding affinity of 10x higher than the other FLT1-ligands [149], was shown to be the highest expressed. In agreement with this, IHC revealed VEGFA expression in all investigated HNSCC tumour samples (11/11, one sample of 12 not available). VEGFB, however, was detected in only 2 of 12 investigated tumours. These data suggest that FLT1 activity in HNSCC may be regulated through VEGFA. Moreover other researchers had already demonstrated that VEGFA silencing results in decreased HNSCC cell survival indeed [73].

We assumed that the level of VEGFA expression per HNSCC cell line could better predict radiotherapy resistance than FLT1 expression in our HNSCC cell lines. To test this hypothesis, we examined the level of VEGFA expression in a panel of 5 HNSCC cell lines. The level of autocrine VEGFA production showed a very tight positive correlation towards radioresistance (Fig. 8B, Gamma = 1, $p<0.01$). In accordance with these observations, other groups already have shown that higher VEGFA expression in HNSCC patient samples predicts for worse local control after radiotherapy [71,72].

4. Conclusion

In the former chapter we identified FLT1 as a TK potentially conferring resistance to radiotherapy and survival in our two HNSCC cell lines SQD9 and SCC61. In this chapter we confirm this assumption indeed to be a valid target. First we confirmed expression of this TK in both the HNSCC cell lines used in the screen. Further we did

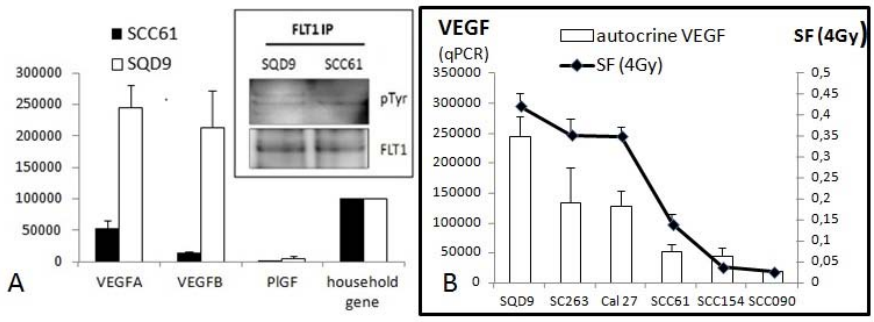


Figure 8: ***Self contained activation through autocrine production of FLT1 ligands is documented in HNSCC, and results in radiotherapy resistance.*** *(A, inset) FLT1 immunoprecipitation (FLT1 IP) showing tyrosine phosphorylation (pTyr) of FLT1 in HNSCC cell lines SQD9 and SCC61. The lower panel shows detection of FLT1 in immunoprecipitates using anti-FLT1 antibody. (A) Autocrine production of FLT1 ligands VEGFA, VEGFB and PlGF is examined by qPCR in SCC61 and SQD9. Data are shown as the averaged means of 3 separate experiments ± SEM after normalization to 100000 copies of household gene HPRT. (B) Radiotherapy resistance measured by clonogenic assay as well as basal autocrine VEGFA production measured by qPCR, in a panel of 5 different HNSCC cell lines. For qPCR data are normalized to 10^5 copies of house hold gene HPRT. Data are presented as the averaged normalized means ± SEM calculated from three separate experiments. SF, survival fraction. A highly significant correlation was noted between cell survival after radiotherapy (4 Gy) and autocrine VEGFA production (Gamma=1, p<0.01)*

show that FLT1 is ubiquitously overexpressed in primary HNSCCS tumor biopsies. Very interestingly, this overexpression seems to be tumor specific, holding great promise for possible therapeutic targeting. By demonstrating that TK knockdown mimics the effect of the shRNAmir using 2 non-overlapping siRNA sequences, we confirm that the induced radiosensitivity and decrease in survival is an effect mediated through FLT1 itself, and not by some off-target effect of the shRNAmir.

Finally, we show that FLT1 is spontaneously phosphorylated in HNSCC cell lines suggesting receptor activation in these cells. Interestingly we find autocrine production of FLT1 activating ligands like VEGFA in these cells as well as in primary HNSCC tumor biopsies. The level of autocrine production of these ligands further seems to correlate with radioresistance, which is an interesting observation, suggesting possible usefulness in the context of predictive biomarker analysis. In a next chapter we examine different possible treatment strategies applicable for clinical FLT1 receptor targeting, and examine why some strategies are effective and others fail.

CHAPTER V

FLT1 TARGETED THERAPY IN HNSCC

Part of this work has been published as:

EJ. Van Limbergen, P. Zabrocki, M. Porcu, E. Hauben, J. Cools, S. Nuyts. FLT1 kinase is a mediator of radioresistance and survival in Head and Neck Squamous Cell Carcinoma. *Acta Oncologica, 2013; Early Online 1-9.*

1. Introduction

Previously FLT1 was demonstrated to be selectively overexpressed in HNSCC. Further it was shown to confer resistance to radiotherapy, as well as improving survival. Interestingly, literature suggests that FLT1 kinase function is expendable for normal life and development [151]. Therefore, as FLT1 is holding great promise as a potential therapeutic target, the exploration of different FLT1 targeting approaches would be of great interest to the oncologic community.

We showed that the FLT1 receptor is activated. This seemed not due to activating mutations but as a consequence of autocrine production of mainly 'FLT1-activating VEGFA'. Theoretically, several FLT1 targeting strategies would be applicable in this case. Sequestration of activating ligand would be a good option, but also receptor blocking molecules interfering with ligand binding would be expected to be effective. On the other hand the use of TKI's would also be an attractive strategy.

Interestingly, as earlier described in breast cancers cells, FLT1 has been shown not to be localized on the plasma membrane as one would expect. Instead it was shown to have an intracellular localization [154]. If the same would be true in HNSCC then plasma permeability would be a very essential characteristic for an inhibitor to successfully interfere with FLT1 signal transduction (see further).

2. Materials and methods

2.1. Cell lines

Two HNSCC cell lines were used: SQD9 and SCC61. Cells were grown in DMEM containing 10% fetal bovine serum (FBS). Cells were maintained at 37° Celsius in a humidified incubator with a 5% CO_2/95% air atmosphere.

2.2. FLT1 Inhibitors

The TKI's sunitinib and axitinib with documented anti-FLT1 activity were obtained from Selleck Chemicals [158,159]. The VEGFA scavenging monoclonal antibody

Bevacizumab which is known to block VEGFA-FLT1 interaction [160] was from Roche. The FLT1 blocking peptide (GNQWFI) [161] was synthesized by Eurogentech.

2.3. Western blotting

Cells were lysed on ice with chilled cell lysis buffer (Cell Signaling) with additional Complete protease inhibitor (Roche) and sodium orthovanadate (6 mM final concentration). Proteins were separated on 4-12% NUPAGE gels (Invitrogen) and were blotted onto PVDF-membranes (Biorad). Membranes were blocked with 5% (w/v) non fatty milk in 1X TBS containing 0.2% Tween-20. Following primary antibodies were used: anti-β-Actin (#4967) anti-KDR (#2479) (Cell Signaling technology). HRP-linked secondary antibodies were used. Detections were performed with an ECL detection system (Perkin Elmer) using an ECL imager (LAS-3000 mini).

2.4. (Confocal) immuno-cytochemistry

For FLT1 immunocytochemistry, cells cultured on autoclaved glass coverslips were fixed in 6% formalin for 15 minutes. Then cells were permeabilized with PBS-tween for 2x5 minutes unless otherwise stated. To minimize aspecific immunoreactivity a blocking step was performed with protein block solution (X0909, DAKO). The primary anti-FLT1 antibody HPA011740 (Atlas Antibodies 1:200) was used. For immunofluorescence the secondary staining was performed with an Alexa-488 coupled Fab fragment (1/1000, Cell Signaling) and counterstaining of the nucleus and cytoskeleton was done with TO-PRO3 (1µM, Invitrogen) and Alexa-555 coupled phalloidin (165nM, Invitrogen) respectively. Fluorescent images were acquired using a Leica SP5 laser scanning confocal microscope. For regular immunocytochemistry, secondary staining was performed with a biotinylated goat anti-polyvalent antibody together with HRP-linked streptavidin (Thermo Scientific). Diaminobenzidine complex (DAB) was used as chromogen. Primary antibodies were omitted in the negative controls.

2.5. Sulphorodamine B (SRB) Survival assays

Cells were seeded one day before treatment. Cells were pre-treated for 72h at the indicated concentration of a specific inhibitor. Then survival was measured using an SRB-assay, as previously described [157]. In short, cells were fixed after treatment with trichloric acid and stained with sulfhorodamine B (SRB). The incorporated SRB was liberated from cells in a TRIS-base solution, and optical densities at 570 nm were determined. Cell survival was determined as the relative absorption of SRB in treated wells in comparison to controls.

2.6. Clonogenic assays

Experiments were carried out as previously described [157]. Cells were treated (24 hours) and irradiated in culture flasks containing the inhibitor, and subsequently trypsinized, counted and seeded 4-6 hours after radiotherapy. The plating efficiency (PE) was defined as the ratio of cells forming a colony of more than 50 cells over the amount of cells seeded, after an incubation period of 2-3 weeks without radiation. The survival fraction was calculated after normalizing the amount of colonies formed after having received a certain dose of radiotherapy to the plating efficiency. The dose enhancement factor was calculated as the ratio of irradiation dose needed in the control condition/irradiation dose needed in the experimental condition to obtain a survival fraction of 0.5.

3. Results

3.1. FLT1 is not located on the plasma membrane but on the inside of HNSCC cells.

For FLT1, a very unique mechanism of intracrine activation was described earlier in breast cancer cells by Lee et al. [154]. Here the authors showed that the FLT1 receptor was not located at the plasma membrane, where one would normally expect a tyrosine kinase to be, but on the inside of the cell on the nuclear envelope. There FLT1 was shown to mediate cell survival of breast cancer cells, a process which was shown only to be affected by autocrine intracellular (intracrine) VEGFA and not

exogenous or extracellular VEGFA. To see if this was also true for HNSCC cells, the subcellular localisation of FLT1 was investigated by confocal microscopy using a FLT1 antibody with confirmed specificity on formalin fixed HNSCC cellular material (Fig. 6A-C). We could indeed confirm intracellular location for FLT1 in HNSCC (Fig. 9A-C). In addition, we used this FLT1 specific antibody, which is raised against the extracellular part of the receptor in another confirmatory assay. If the FLT1 receptor would be localized on the plasma membrane, one would expect FLT1 immunoreactivity to be present even when immunocytochemistry is performed without permeabilising the plasma membrane. We showed this not to be the case since omission of the permeabilising detergent Tween resulted in nearly complete absence of immunoreactivity (Fig. 9D-I).

3.2. Therapeutic targeting of the FLT1 receptor: a need for membrane permeable inhibitors

If FLT1 driven HNSCC cell survival and radioresistance is an intracellular process one would expect only membrane permeable inhibitors to be useful for therapeutic targeting. Two strategies using membrane impermeable inhibitors were used: a FLT1 blocking peptide (GNQWFI) preventing ligand binding to the FLT1 receptor [161], and the VEGFA blocking monoclonal antibody bevacizumab [160]. None of both strategies was shown to affect HNSCC cell survival (Fig. 10C-D). On the other hand we tested two membrane permeable tyrosine kinase inhibitors with documented FLT1 activity: axitinib and sunitinib [158,159]. Both inhibitors were effective in reducing cell survival of SCC61 and SQD9 cells in a dose dependent manner (Fig. 10A-B). A 72h treatment with 2uM axitinib resulted in reduction of cell survival to 67% and 50% in SQD9 and SCC61 respectively, whereas sunitinib at 2µM concentration reduced cell survival to 39% and 51% (all $p<0.05$). Importantly, western blot and q-PCR analysis showed absence of KDR expression in these cell lines, excluding the possibility that these inhibitors induced their cytotoxic through KDR effects in a FLT1

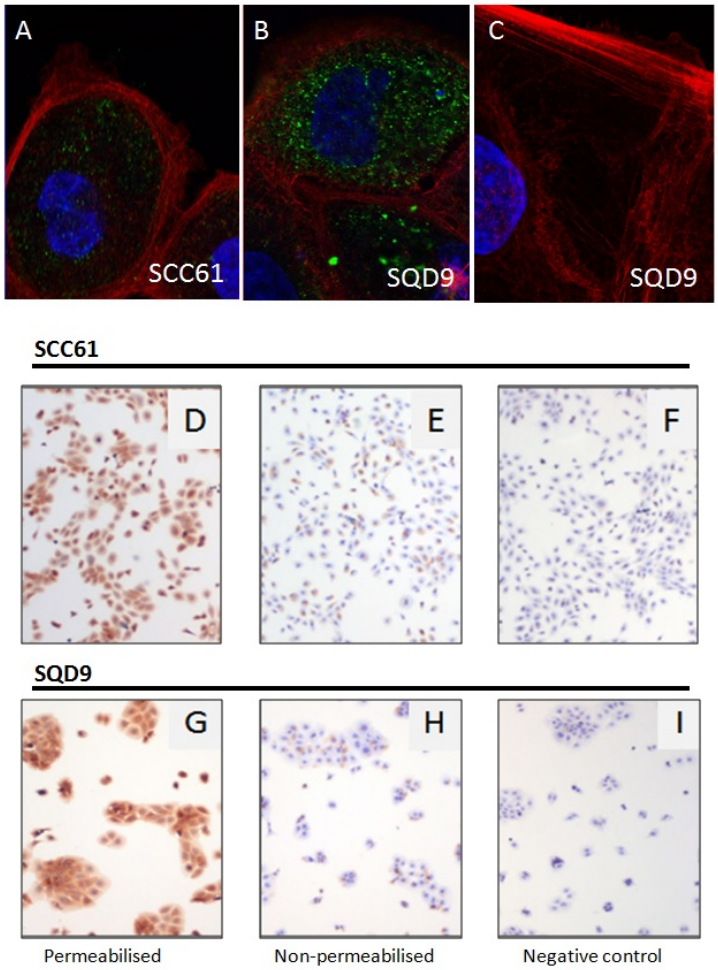


Figure 9: ***FLT1 has an intracellular localization.*** *(A-C) Merged color confocal microscopy images taken from SCC61 (A) and SQD9 (B). FLT1, shown in green is located intracellularly. Fiber-actin (Phalliodin-Alexa555) and nucleus (To-Pro3) are shown in red and blue respectively. (C) FLT1 negative control staining where only a secondary Alexa 488 coupled Fab fragment was used. Scale bars 10µm. (D-I) Classical immunocytochemistry using a FLT1 antibody against the extracellular part of the FLT1 receptor (x40). SCC61 (D) and SQD9 (G) show strong immunoreactivity when cells are permeabilized with Tween. However, when Tween permeabilisation is avoided, immunoreactivity in SCC61 (E) and SQD9 (H) becomes comparable to their respective negative controls (F, I).*

independent manner (Fig. 10G). Finally, we tested the effectiveness of axitinib as a radiosensitizer in a clonogenic assay on SCC61 and SQD9 cells. Axitinib was indeed effective in inducing radiosensitization (Fig. 10E-F). Comparable to what we observed after siRNA mediated FLT1 silencing, the radiosensitizing effect of axitinib (2 µM

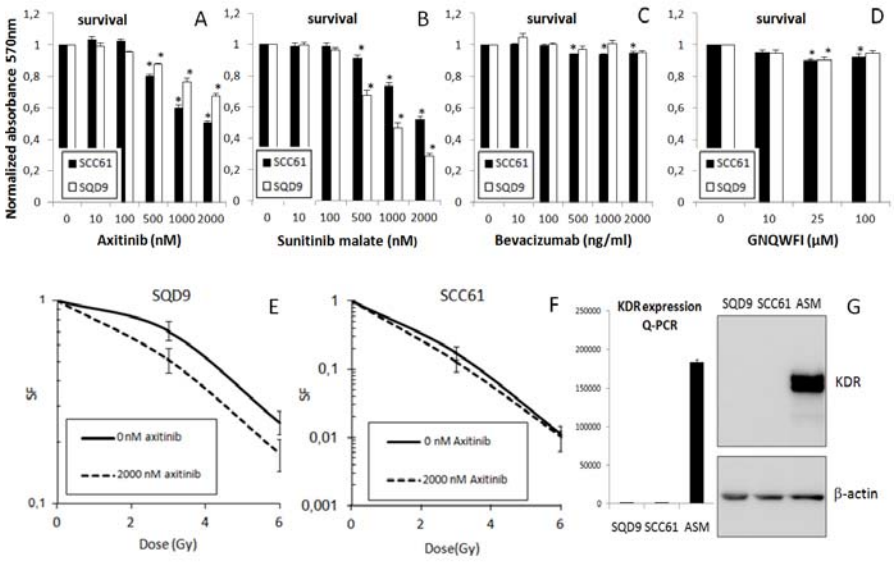


Figure 10: ***The effect of different FLT1 targeting strategies on cell survival and radioresistance.*** *(A-D) Cell survival measured with an SRB assay using indicated concentrations of axitinib, sunitinib, bevacizumab and GNQWFI respectively. Data are presented as averaged normalized means ± SEM calculated from three separate experiments. *, significantly different for the respective controls, p<0.05, students t-test. (E-F) Clonogenic assays investigating the radiosensitizing potential of axitinib in SQD9 and SCC61. (G) Q-PCR (left) and western blot data (right) demonstrating KDR is not expressed in SQD9 and SCC61, excluding that axitinib and sunitinib induce their cytotoxic or radiosensitizing effects through KDR. Angiosarcoma (ASM) cDNA and protein lysates were used as positive controls for KDR expression. For qPCR, expression is shown as the amount of KDR per 10^5 copies of HPRT. β-actin is used as loading control for western blot. Data shown are the averaged means of at least three independent experiments ± SEM. SF, survival fraction.*

concentration) was clearly more pronounced in the more radioresistant HNSCC cell line SQD9 (Fig. 5E-F, chapter IV) that produces higher levels of FLT1 activating VEGFA (Fig. 8C, chapter IV). The dose enhancement factors we found for axitinib (1.73 for SQD9 and 1.24 for SCC61) were comparable to these found for FLT1 siRNA (1.62 for SQD9 and 1.20 for SCC61; see Chapter IV 3.1).

4. Conclusion

FLT1 tyrosine kinase is known as a suppressor of angiogenesis [149,150]. In this context, it was shown that FLT1 effectuates this function not through its kinase domain but rather through its extracellular region by trapping VEGFA. In this way FLT1 prevents stimulation of angiogenesis through the VEGFA-VEGFR-2 receptor complex. In HNSCC however, we believe FLT1 fulfils its function as oncogenic driver through its tyrosine kinase activity, not only by showing that the FLT1 receptor kinase function is activated, and but also by demonstrating that FLT1 tyrosine kinase inhibitors are able to downregulate HNSCC survival and radioresistance.

In breast cancer cells oncogenic FLT1 signalling has been shown to be an intracrine process: FLT1 is located on the inside of breast cancer cells where it can only be activated by autocrine produced intracellular VEGFA. The observations we made on HNSCC cells are comparable as we document FLT1 to be located only intracellularly in HNSCC. Further we document that only plasma membrane permeable FLT1 inhibitors are able to affect HNSCC cell survival and radioresistance. In practice this intracrine signaling severely limits therapeutic options since its means that currently only tyrosine kinase inhibitors can be used. To date however, no specific FLT1 kinase inhibitor exists. In fact, the currently available candidates are mostly inhibitors designed as anti-angiogenic therapy against KDR with additional anti-FLT1 activity. This is also the case for the inhibitors axitinib and sunitinib used in this study. Although some data are available for FLT1 inhibitor effectiveness from *in vitro* assays, it is currently not known if tumoral FLT1 can be inhibited using these inhibitors *in*

vivo. Therefore we believe these data should be taken as a proof of concept that encourages the development of better and more specific FLT1 inhibitors.

CHAPTER VI

HPV ASSOCIATED p16 UPREGULATION IMPAIRS AUTOCRINE VEGFA EXPRESSION, BUT DOES NOT AFFECT HYPOXIA TOLERANCE

Part of this work has been presented at ESTRO 31in an oral presentation as:

EJ. Van Limbergen, R. Dok, E. Hauben, S. Nuyts. P16 in head and neck cancer: a marker of impaired HIF1α function and decreased hypoxia tolerance? ESTRO31, 2012 May, Barcelona Spain .

1. Introduction

We discovered FLT1 to be a general driver of HNSCC radioresistance. Moreover the FLT1 ligand VEGFA correlates with radioresistance *in vitro* as well as *in vivo* (Chapter III.3.3). However how these finding correlate to HPV status in HNSCC remains unclear. As an offset to answer this question we started from observations done by Zhang et al. showing that p16 suppresses the expression of the FLT1 activating ligand VEGFA, through inhibition of the key regulator of hypoxic metabolism HIF1α. If it could be shown that p16 is suppressing HIF1α in HPV positive HNSCC, the resulting hampered VEGFA expression maybe could explain part of the increased radiosensitivity through impaired FLT1 activity. Further, an impaired HIF1α function could lead to hypoxia intolerance, owing to impaired adaptation to cellular metabolic stress as a result of oxygen deprivation. This hypoxia intolerance would then lead to a severe impact on cellular survival under oxygen deprivation, and therefore lead to increased radiosensitivity in vivo. Further it could also explain the clinical findings reporting no effect of hypoxia targeting specifically in HPV-related HNSCC (see discussion in chapter I.4.2.2.5).

2. Materials and methods

2.1. Cell lines and reagents

Six HNSCC cell lines were used: SQD9, SCC61, SC263, Cal27, SCC090 and SCC154.

HPV status was confirmed by a PCR reaction using the GP5+/6+ primer set [162] and p16 western blot (see further). SQD9, SCC61 and SC263 were a gift from A.C. Begg (Netherlands Cancer Institute, The Netherlands). Cal-27 was obtained from F. Lardon (University of Antwerp, Belgium). HPV positive HNSCC cell lines SCC090 and SCC154 were obtained from S. Gollin (Pitsburg) and DSMZ [163] respectively. SQD9, SCC61, SC263, Cal27 cells were grown in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco). SCC090 and SCC154 cells were cultured in MEM (Gibco) containing 10 % FBS, 1% MEM Non Essential Amino Acids (Gibco) and 1% L-glutamine (Gibco).

Cobalt Chloride (CoCl2), used as a HIF1α inducer, was obtained from SIGMA and used at 250 μM concentration.

2.2. *RNA interference*

Transfection was performed using Lipofectamine 2000 (Invitrogen). Stealth select siRNA's targeting p16 were obtained from Invitrogen (sequences AGAACCAGAGGCUCUGAtt, and CGCACCGAAUAGUUACGGUtt, designated as p16A and P16B respectively). Transfection was performed using 100 pMoles of siRNA, and 5 μm of Lipofectamine 2000 (Invitrogen) per 600.000 cells in 2 ml of growth medium. Transfection efficiency was monitored using Block-it Green Fluorescent siRNA and flow cytometry. Since insufficient transfection efficiency could be achieved using the standard manufacturers protocol, the transfection procedure needed to be adapted. Eventually it appeared that transfection efficiency of wells SCC154 cells was dramatically improved by *not* allowing these cells to attach to the culture plates before the transfection procedure.

Lentiviral shRNAs against luciferase or p16/INK4A were purchased from Sigma Aldrich. Lentiviral infections were performed as previously described [164], and infected cells were selected by treatment with 4μg puromycin for 4 days. The used p16 targeting sequences were CACTACCGTAAATGTCCATT and CCGATTGAAAGAACCAGAGA (designated as shp16A and shp16B respectively).

2.3. Western blotting

Cells were lysed on ice with chilled cell lysis buffer with additional Complete® protease inhibitor (Roche). Proteins were separated on 4-12% NUPAGE gels (Invitrogen) and were blotted onto PVDF-membranes (Biorad). Membranes were blocked with 5% (w/v) non fatty milk in 1X TBS containing 0.2% Tween-20. Following primary antibodies were used: anti-p16 (Cell Signaling, #4842) and anti-HIF1α (610958 BD Pharmigen) and anti-GAPDH (Sigma, G9545). HRP-linked secondary antibodies were used. Detections were performed with an ECL detection system (Perkin Elmer) using an ECL imager (LAS-3000 mini).

2.4. Quantitative real-time PCR

Experiments were carried out on a Lightcycler 480 II (Roche) in a reaction volume of 15 µl containing 5µl of diluted cDNA, 7.5 µl SYBR green reaction mix (Roche), 1.25 µl of 5µM forward primer and 1.25 µl of 5µM reverse primer. Quantification was performed using the $\Delta\Delta$Ct method. Primers were designed using LightCycler Probe Design2 software. Used primer sequences are listed in Supplementary table 2 . The PCR cycle conditions were as follows: a preincubation step for 10 minutes at 95°C followed by 40 amplification cycles; each cycle included 10 seconds at 95°C and 30 seconds at 60°C; followed by melting curve analysis to verify specific amplification of the amplified product. Messenger RNA was extracted from cells using the RNeasy mini Kit (Qiagen) with additional 'on column DNA digestion'. cDNA was synthesized using Superscript® VILO (Invitrogen). For experiments involving siRNA mediated knockdown of p16, cells were subjected 48h after transfection to 250µM of CoCl2 for another 12 h before mRNA extraction. For experiments involving shRNA gene expression experiments, mRNA was extracted after 24h of hypoxia (1% O2) exposure.

Table 2: REAL TIME-qPCR PRIMER sequences

Gene	Primer sequence
VEGFA	**F:** 5'- CCATGCAGATTATGCGGATCA -3'
	R: 5'- GCATTCACATTTGTTGTGCTGTA -3'
GLUT1	**F:** 5'-CTGTGTGGTCCCTACGTC-3'
	R: 5'-CTCAGGAACTTTGAAGTAGGTG-3'
LDHA	**F**: 5'-TTCCACCATGATTAAGGGTCTTTAC-3'
	R: 5'-AGGTCTGAGATTCCATTCTGT-3'
PDK1	**F:** 5'-CACCAAGACCTCGTGTTGAGA-3'
	R: 5'-GCGTAAAGACGTGATATGGG-3'

2.5. Immunohistochemistry and HPV-testing in patient samples

FFPE tumor samples were obtained from patients diagnosed with squamous cell carcinoma of the oropharynx between 2000 and 2010. Deparaffinisation and antigen retrieval of 5 µm sections was performed using an automated protocol on a DAKO PT link module. The following primary antibodies were used: anti-VEGFA (1:100, 30' room temperature; SC-152, Santa Cruz), anti-HIF1α (1/500, overnight at 4°C, BD Pharmigen, 610958) and anti-p16 (1/80, room temperature, BD Pharmigen, 550834). Secondary antibody detection was performed with a horseradishperoxidase coupeled secondary antibody. Diaminobenzidine complex (DAB) was used as chromogen. Counterstaining was performed with hematoxylin. A sample was considered as p16 positive when at least 50% of cells showed clear p16 immunoreactivity. A sample was considered HIF1α positive according to the presence or absence of nuclear HIF1α immunoreactivity in the sample. Because HIF1α expression is inhomogeneously upregulated only in smaller parts of HNSCC tumors, we decided to score VEGFA accordingly to maximal focal immunoreactive intensity, as grade I (lowest intensity) to grade III (highest intensity).

HPV testing was performed by HPV-PCR using the GP5+/6+ primer set [165,166]. From each FFPE block, several sections of 5 micron thickness were cut, using a fresh microtome for each block to avoid DNA carry over. The first and the last section were H&E stained to confirm the presence of tumor in the used biopsies.

2.6. *Cell survival analysis*

In siRNA experiments cells were transfected in 96-well plates and then grown for another 72h in a normal incubator (21% O) before the cells were subjected to hypoxia (1% O2) or normoxia (21% O2) for 72 h. For experiments with shRNA mediated knockdown stable shRNA expressing clones were subjected to 72h of hypoxia (1% O2) or normoxia (21% O2). Cell survival analysis was then performed using an SRB-assay, as previously described [157]. Survival curves were generated by normalizing the

hypoxic survival data to the normoxic survival data, to account for possible differences in growth rate between the different siRNA and shRNA treated SCC154-cell lines.

In inhibitor experiments cells were seeded one day before treatment. Cells were pre-treated for 72h at the indicated concentration of sunitinib. Then survival was measured using an SRB-assay, as previously described [157].

2.7. Metabolite analysis, and growth curves

For metabolite analysis cells were cultured in a normal incubator (21% O2) or in a hypoxia chamber (1% O2) for 24h hours. After 24h, media was collected and immediately frozen until analysis through a lactate dosage kit (from Abcam) according to manufacturer's instructions. Cells were lysed with PBS + 1% Triton-X to measure protein concentration with a BCA assay (Pierce), as a mean to correct for differences in cell quantity. For growth curve analysis cells were seeded in 96-well plates and the amount of cells was measured using an MTT-assay at 0h, 24h, 48h, 72h and 96h.

3. Results

3.1. HPV associated p16 overexpression correlates with lower VEGFA expression, lower FLT1 dependence, and higher radiosensitivity.

When VEGFA expression was evaluated using qPCR among several different cell lines we found our HPV related p16 overexpressing HNSCC cell lines to have the lowest VEGFA expression (Fig. 11A). A lower level of autocrine VEGFA expression could mean that these cells were less FLT1 driven and therefore less radioresistant, as demonstrated previously (see Fig. 8B, Chapter 4). To verify this hypothesis we tested the FLT1 inhibitor sunitinib in a survival assay, since FLT1 was also demonstrated to affect HNSCC survival. Figure 11B indeed shows our p16 positive

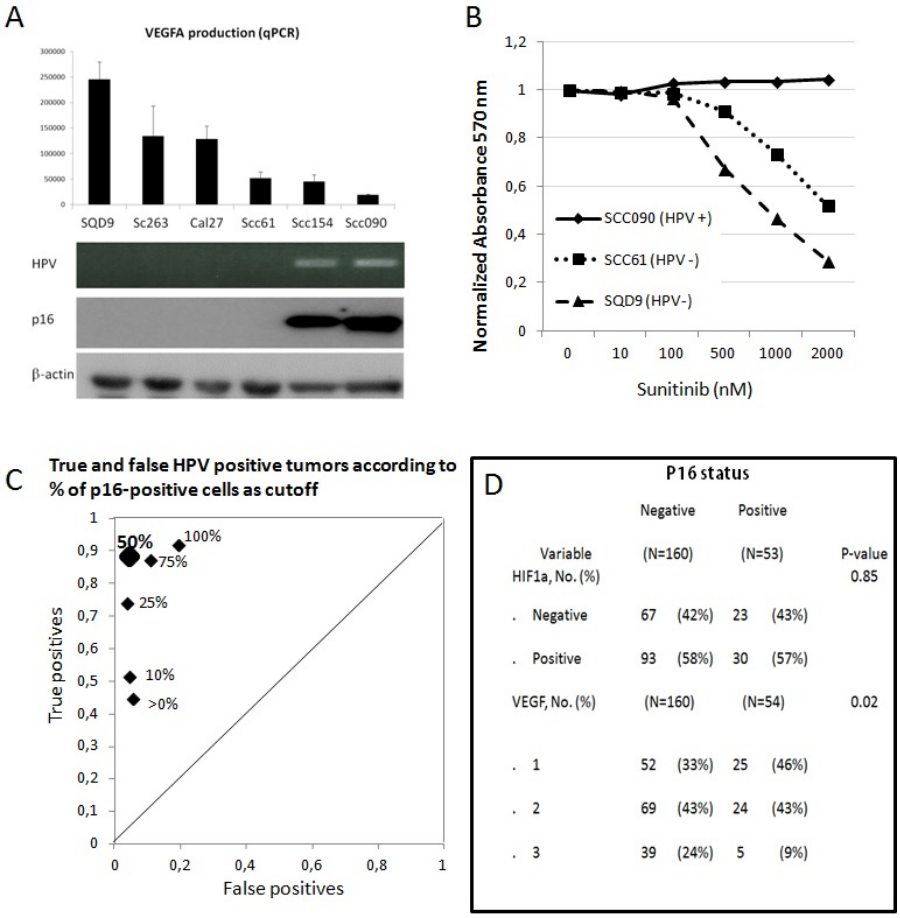


*Figure 11**: HPV associated p16 overexpression correlates with lower VEGFA expression, lower FLT1 dependence.** (A) VEGFA expression analysis in a panel of HNSCC cell lines. HPV(+), p16 positive cell lines have the lowest levels of VEGFA gene expression. (B) Survival measured in different HNSCC cell lines treated with the indicated doses of sunitinib, as measured by SRB-assay. (C) Receiver operating characteristics (ROC) curve showing 50% of p16 positive cells being the optimal cutoff correlating to HPV status using HPV-PCR as golden standard, and taking into account true and false positive rates. (D) Results of VEGFA and HIf1α protein expression as measured by immunohistochemistry. P16 positive cells in general have lower maximal VEGFA expression but similar expression of HIF1α.*

HPV related HNSCC cell line SCC090 did not encounter any decrease in cell survival after FLT1 inhibition, as measured by an SRB assay. IHC experiments on head and neck cancer biopsies were carried out to investigate if p16 would also correlate with decreased VEGFA expression *in vivo*. The cutoff for regarding a tumor as HPV positive was chosen at >50% of cells having positive P16 staining at the cytoplasm, since this cutoff was shown to correlate best with positive HPV status (Fig. 11C). Since hypoxic regions with HIF1α upregulation are a focal phenomenon, VEGFA expression was classified according to maximal focal staining intensity found in the biopsy. Although we found no difference in HIF1α expression in p16 positive vs. negative biopsies, focal maximal VEGFA expression was higher in p16 negative samples, suggesting that the difference in VEGFA expression could not be attributed to an increase in HIF1α expression in p16 negative tumors (Fig. 11D). These data are compatible with the model of HIF1α getting upregulated but being functionally less active because of the possible interaction with p16.

3.2. *P16 suppresses HIF1a activity as a transcription factor in HPV related HNSCC.*

To test the possibility of p16 suppressing HIF1α activity, p16 expression was knocked down in the HPV related HNSCC cell line SCC154 using shRNA (Fig. 12A). To induce HIF1α activity shRNA transfected cells were grown in a hypoxia chamber under 1% oxygen to induce HIF1α expression. The expression of several HIF1α associated genes (VEGFA, LDHA, GLUT-1, PDK-1 [139]) was subsequently tested by qPCR (Fig. 12B-E). These experiments showed indeed that p16 knockdown resulted in a significant increase in HIF1-related gene expression, but only under hypoxia where HIF1α becomes upregulated.

It is however important to note that these experiments also showed some increase in HIF1α regulated gene expression after HIF1α upregulation by exposure to hypoxia in the condition without p16 knockdown, meaning that in HPV-related HNSCC the p16-related HIF1α suppressive activity is not completely effective (Fig. 12B-E).

To confirm these findings experiments were repeated using an siRNA mediated setup, where this time CoCl2 was used as a HIF1α inducer (Fig. 13A). The findings were similar as those with the shRNA under hypoxia (Fig. 13B-D). Importantly the siRNA sequence p16B has no sequence overlap with p14ARF, excluding that the higher described effects could be attributed to knockdown of p14ARF, an alternate reading frame product of the p16 gene focus CDKN2a.

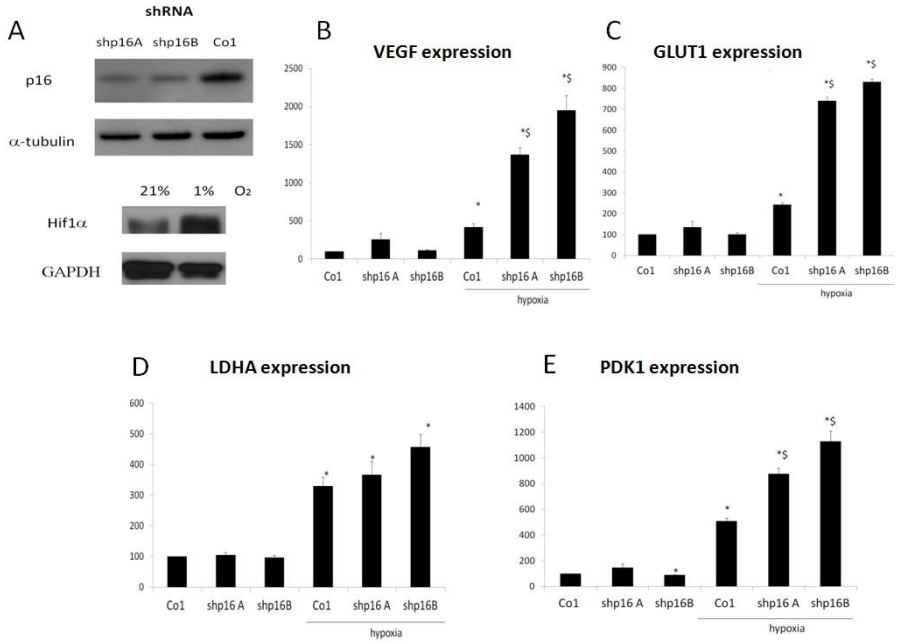


Figure 12: ***ShRNA mediated pP16 knockdown increases HIF1α mediated gene expression in the HPV positive cell line SCC154.*** *(A) Western blot demonstrates p16 knockdown using shRNA vectors in SCC154 as well as HIF1α upregulation during 24h of exposure to hypoxia (1% O2). (B-E) Q-PCR gene expression analysis of the HIF1α regulated genes VEGFA, GLUT1, LDHA and PDK1). Data are presented as the percentage of expression relative to non silencing control shRNA (Co1) in normoxic conditions. * significantly different from Co1 under normoxia, $ significantly different from Co1 under hypoxia, using a 2-sided students T-TEST.*

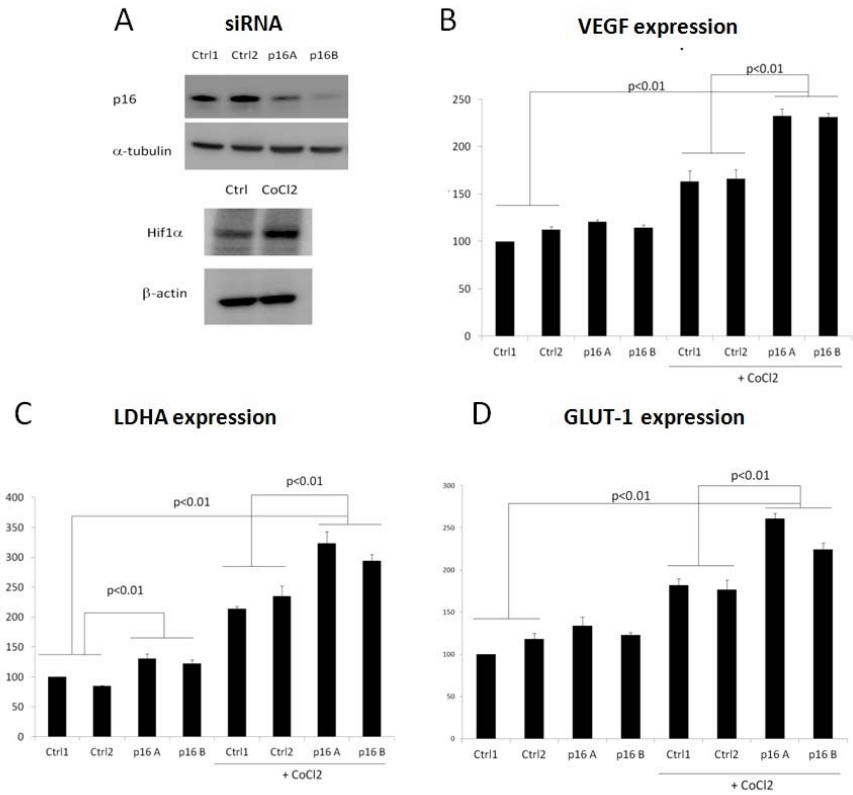


Figure 13: ***: siRNA mediated p16 knockdown increases HIF1α mediated gene expression in the HPV positive cell line SCC154.*** *(A-D) Data obtained with shRNA are confirmed using a setup using siRNA mediated p16 knockdown. (A) Western blot demonstrates p16 knockdown using siRNA designated as p16A and p16B, as well as HIF1α induction by CoCl2 (250 µM). (B-D) Q-PCR gene expression analysis shows improved gene expression of HIF1α regulated genes VEGFA and LDHA after p16 knockdown and HIF1α induction by CoCl2. Data are shown as percentage of the averaged means of 3 separate experiments ± SEM after normalization the control condition (using Ctrl1 siRNA). P-values were calculated using a 2-sided students T-TEST.*

3.3. P16 related HIF1α suppression does not affect survival under hypoxia.

Higher described data show that in HPV related HNSCC cells, p16 is able to suppress HIF1α activity, although not completely. The next question was whether this suppression was sufficient to affect hypoxia tolerance. P16 was knocked down using siRNA or shRNA in the SCC154 cell line. Cells were then cultured in 96-well pates under normoxia as well as hypoxia. Survival was then measured by means of an SRB assay and survival under hypoxia was normalized to survival under normoxia to account for changes in normal growth rate after gene silencing. Both with siRNA as well as shRNA there was no survival benefit seen under hypoxia after p16 silencing (Fig. 14A-B).

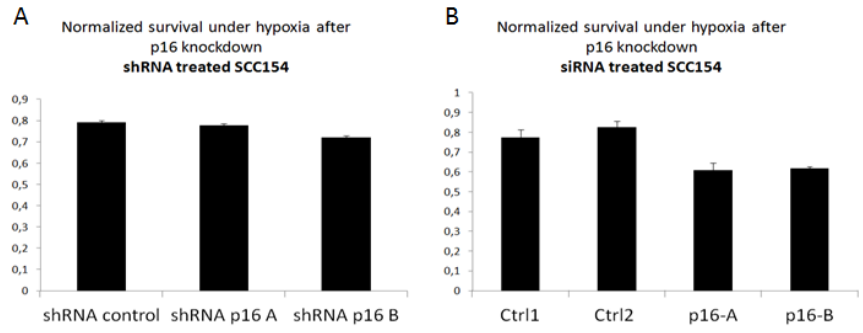
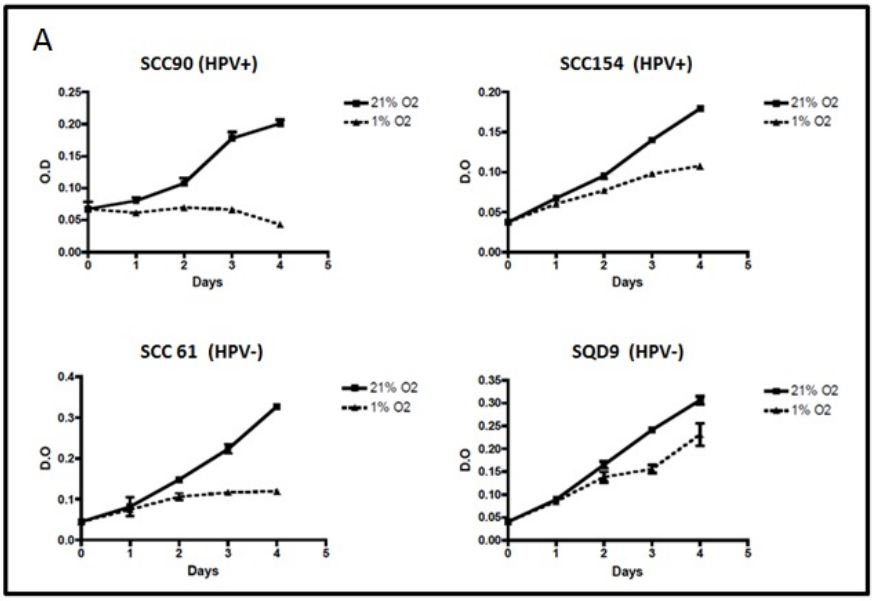


Figure 14: ***p16 knockdown does not improve cell survival under hypoxia.*** *Survival of SCC154 cells is not improved after shRNA mediated (A) or siRNA mediated (B) p16 knockdown. Bars show the survival rate +/- SEM of cells under hypoxia relative to the survival of the same knockout condition under normoxia. This was done to account for possible differences in growth rate after exposure to different shRNA's or siRNA's.*

3.4. HPV related HNSCC cell lines are able to increase glucose usage, induce lactate metabolism and sustain hypoxic stress.

P16 knockdown did not improve survival under hypoxia. Therefore we assessed the hypoxic survival potential of 2 HPV related cell lines (as well as 2 HPV unrelated cell



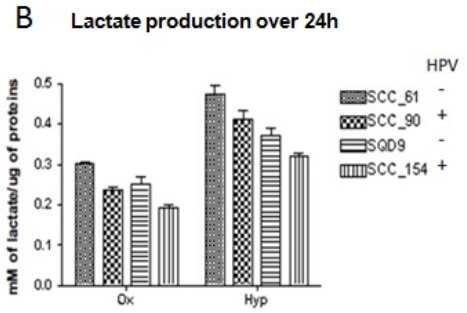


Figure 15: ***HPV positive cells are able to survive under hypoxia.*** *(A) MTT based growth curves show HPV(+) HNSCC cell lines are able to survive under hypoxia, just as HPV(-) HNSCC cells. (B) Metabolite analysis shows that HPV(+) cells can make a glycolytic metabolic shift towards more lactate production, like HPV(-) HNSCC cells.*

lines) to confirm if these HPV related cells were indeed able to survive in hypoxic conditions. We noted that the HPV-related cell lines were able to survive for a prolonged period of time under hypoxia, like HPV-unrelated cells (Fig. 15A). HIF1α is able to change metabolism to a more glycolytic phenotype. These changes are associated with extracellular acidification, higher glucose use and lactate production [139]. Additionally, we documented that HPV-positive HNSCC cell lines were, just as HPV-negative cell lines able of making a glycolytic shift in their metabolism toward a more glycolytic type, by increasing lactate production (Fig. 15B).

5. Conclusion

In this chapter, we attempted to shed light on the molecular basis of the increased radiosensitivity of HPV related HNSCC cells by assessing the impact of HPV associated p16 overexpression on VEGFA expression as well as tumor hypoxia tolerance through a negative regulatory impact on HIF1α, the key regulator of hypoxic metabolism [167].

Our series of experiments however, showed that HPV related tumors are able to switch their metabolism towards a glycolytic phenotype and that these tumor cells are able to survive under an oxygen level of 1% (or 7.6 mmHg). However, while cells surviving at this oxygen tension are clearly protected from the cytotoxic effects of ionizing radiation compared to 21% oxygen level (or 159.6 mmHg) the radioprotective effect of hypoxia has been shown to increase further to values of 0.07% oxygen level (or 0.5 mmHg) [24].

Moreover oxygen tensions below 1% (7.5 mmHg) certainly occur in HNSCC [168]. Therefore our data obtained from *in vitro* experiments cannot completely rule out that hypoxia intolerance in HPV-related cancer cells occurs *in vivo*, where the conditions are even more grim.

We did clearly find evidence of a negative impact of p16 on HIF1α functioning although this impact was not sufficient to prevent a metabolic switch. Possibly here, the HIF1α stimulating role of the HPV related oncogenes E6 and E7 becomes

extremely important [169]. Because of the disruption of the RB pathway as a consequence of viral E7-expression, the cyclin dependent kinase inhibitor p16 gets upregulated and starts suppressing HIF1α. A stimulatory role of HIF1α, by E6 and E7 would then be necessary to counterbalance the HIF1α inhibitory activity of p16 to allow VEGFA expression, angiogenesis and tumor formation.

Our *in vitro* and *in vivo* data indeed are all compatible with a negative impact of HPV-associated p16 overexpression on HIF1α resulting in a decreased VEGFA expression. Therefore it is very plausible that decreased FLT1 stimulation in HPV positive HNSCC in part explains the increased radiosensitivity of these tumors, next to other patient related factors, immune response or DNA-repair (see Chapter I.4.2).

A possible association between VEGFA and HPV/p16 positive status in HNSCC was also investigated in other series but there no association was confirmed [170,171]. However one must note that in these studies, VEGFA scoring was done differently, reflecting a more general staining pattern, and therefore possibly correlating not as well to our method of scoring which was focusing on maximal focal staining mimicking HIF1α stain patterns.

CHAPTER VII

EPIDEMIOLOGIC ASPECTS OF HPV RELATED HNSCC IN FLANDERS (BELGIUM)

This work is currently 'in press' in B-ENT as:

EJ. Van Limbergen, E. Hauben, F. Duprez, D. Van den Weyngaert, M. Voordeckers, W. DeNeve, S. Nuyts. HPV related Oropharyngeal cancers in Flanders (Belgium): Results from a large multicenter study

1. Introduction

Human Papilloma Virus (HPV) has recently been attributed as a major etiologic cause of oropharyngeal carcinoma. Studies suggest large geographic differences exist regarding prevalence of this disease [95]. However until now, we have no idea about the role of HPV in Belgian/Flemish oropharyngeal cancer patients. However, this information might have important implications for the patient:

-Prevention: As there is already a marketed vaccine offering protection against HPV-associated cervical cancer, there is speculation that it may also offer protection against the subset of oropharyngeal cancers that are HPV-associated.

If our data show high incidences of high-risk HPV-associated cancers in men, this might suggest that vaccination of all adolescents against this oncogenic risk should be considered. Prospective studies should be designed to assess the effectiveness of HPV vaccines for the prevention of these cancers.

-Therapy adjustment: The association between HPV and head and neck cancers is already changing the way clinical trials are designed, with investigators stratifying patients by HPV status. New therapeutic trials will have to be designed specific for HPV-positive or HPV-negative patients because of the differences in treatment response in the two groups. Less intensive treatment schedules will have to be looked at. The link between HPV and oropharyngeal cancer also raises the possibility of targeting those tumours with a therapeutic vaccine.

-Follow up: Although HPV-associated oropharyngeal cancer has a relatively good disease-free survival rate, a subset of persons develops recurrence of their cancer. A screening test that takes advantage of a unique marker that is associated with HPV infection could be beneficial for the detection of persistent disease or early recurrence. Salivary rinsing and detection of HPV E6 or E7 copy number could be of value.

-Future translational research: Molecular mechanism underlying the differences in treatment response can lead to new therapeutic opportunities. Further studies are needed to show whether oral HPV infection progresses to dysplasia, as it does in cervical cancer. Doing so would take a prospective, longitudinal study of people with HPV infection, followed by visual detection of premalignant changes or looking for surrogate markers of progression by using random biopsies.

A multicenter cooperative study was undertaken between the radiation-oncology departments of the Flemish universities.

2. Materials and Methods

2.1. *Subjects*

The study was a joint effort of the Flemish radiation oncology centers of Antwerp, Brussels, Gent and Leuven. All patients registered with an oropharyngeal carcinoma were included, whether or not they eventually received radiotherapy. Other inclusion criteria were squamous cell carcinoma histology, and from all patients FFPE tumoral tissue had to be available. Patients were diagnosed between 2000 and 2010.

From these patients follow-up data was collected consisting of identification number, date of birth, gender, tobacco and alcohol usage as noted in the medical records, sublocalisation in the oropharyngeal subsite, TNM and tumor stage (UICC 7th edition), type of therapy received, start and end of treatment received, date of local or regional relapse, date of distant recurrence, date of death and date of last follow-up. For survival analysis, only patients irradiated on macroscopic tumor, receiving ablative doses of radiotherapy ($\geq$ 60Gy) were included. Patients treated with the intent of giving ablative doses but where radiotherapy was canceled due to tumor progression during radiotherapy were not censored.

2.2. *Tumor samples and laboratory studies*

For all patients FFPE tissue was centralized (Leuven) for HPV-status determination. HPV testing was performed using a previously validated algorithm using p16

immunohistochemistry (IHC) followed by HPV-PCR [165,166]. A tumor was regarded as HPV related when both P16 IHC as well as HPV-PCR were positive.

From each FFPE block, several sections of 4 micron thickness were cut, using a fresh microtome for each block to avoid DNA carry over. The first and the last section were H&E stained to confirm the presence of tumor in the used biopsies. A third slice was used for p16 immunohistochemistry, another 3 slices were used for DNA extraction and PCR testing.

Deparaffinisation and antigen retrieval and IHC were performed using an automated protocol on a DAKO PT link module and a DAKO autostainer. For p16 IHC a purified mouse anti-human p16 antibody (G175-405, BD Pharmigen) was used. Sections were scored as P16 positive when clear p16 immunoreactivity was seen in at least 50% of cells [15,172].

DNA was extracted from PPFE sections using the QIAamp DNA FFPE Tissue kit. Concentration and purity were then defined with spectrophotomety (Nanodrop ND-1000). The quality of the extracted DNA for usage as a PCR-template was verified by generating a 167 bp fragment of the IDH2 gene using PCR. Subsequently HPV status was determined with a PCR reaction using the GP5+/6+ primer set capable of detecting at least 16 other high risk-HPV subtypes next to the most common subtypes HPV-16 and HPV-18 [162].

2.3. *Statistics*

The confidence interval for HPV prevalence is based on Wilson (score) confidence limits for a binomial proportion. Inverse probability weighting (IPW) was used to deal with non-random missingness of HPV status. Differences between HPV(-) and HPV(+) groups were analyzed using the Chi-square test in case of categorical predictors, whereas the Mann-Whithey U-test was used for ordinal or continuous predictors. Follow-up summary statistics are based on the Kaplan-Meier estimate of potential follow-up. Overall survival was defined as the time between start of radiotherapy and

death. Disease free survival was defined as the time between start of radiotherapy and disease recurrence or death. Locoregional control was defined as the time between start of radiotherapy and the date of locoregional recurrence. Differences between groups regarding survival or other time-to-event outcomes are analyzed using the Log Rank test. Kaplan-Meier estimates were obtained for estimation of survival curves and follow-up. For the analysis of independent predictor effects on time-to-event outcomes, multivariable Cox proportional hazards models were used, including the predictor of interest as well as possible confounders. Changes in incidence rates over time were calculated using the method of Estimated Annual Percentage Change. All analyses have been performed using SAS software, version 9.2 of the SAS System for Windows. All tests are 2-sided and a 5% significance level is considered for all tests.

3. Results

3.1. *Baseline patient characteristics*

In total *264* patients with an oropharyngeal squamous cell carcinoma were included of which medical records and FFPE tissue were obtained. For baseline patient characteristics see table 3. The mean follow-up our cohort was 4.44 years (2.41 years Q1, 6.63 years Q3). The majority of patients were diagnosed at advanced disease stage: *51* (19%) and *192* (72%) patients had UICC stage III and IV disease. The median age of the study population was 58.8 years, ranging from 26 to 83 years. The most frequent tumor localizations in the oropharyngeal subsite were tonsillar and base of tongue (BOT), 114 (43%) and 88 (33%) of patients respectively. Of *264* patients, *198* (75 %) received and ablative doses of radiotherapy ($\geq$ 60Gy) on macroscopic tumor. Four (2%) patients did not receive radiotherapy. Concurrent chemotherapy was given to *152* (58%) patients. Seven patients (3%) received concurrent treatment based on EGFR inhibition, and *100* patients (38%) received radiotherapy only. The median overall treatment time was 44 days (Q1 42.0 days, Q3 47 days). Adequate

information regarding smoking and ethanol use was retrieved from the medical files for *230* (87%) and *189* (72%) of *264* patients.

Table 3: Patient characteristics

Characteristic	**All patients (*N*=264)**	**HPV neg. (*N*=196)**	**HPV pos. (*N*=53)**	***p*-value** [$]
HPV status, N (%)				
. Negative	196 (74%)	196	/	
. Positive	53 (20%)	/	53	
. Unknown*	15 (6%)	/	/	
Gender				
. Male	211 (80%)	159 (81%)	41 (77%)	0.73
. Female	53 (20%)	37 (19%)	12 (23%)	
Age, Median (Q1, Q3)	58.8 (52.2, 65.8)	58.0 (51.3,65.2)	62.1 (53.3, 71.1)	0.028
UICC-stage number, *N* (%)$				
. I	5 (2%)	4 (2%)	1 (2%)	0.026
. II	16 (6%)	14 (7%)	2 (4%)	
. III	51 (19%)	43 (22%)	5 (9%)	
. IV	192 (73%)	135 (69%)	45 (85%)	
Smoking, *N* (%)				
. Never	28 (112%)	11 (6%)	14 (31%)	<.001
. Stopped >1 year	35 (15%)	22 (13%)	8 (18%)	
. Current	167 (73%)	139 (81%)	23 (51%)	
. Unknown	34	24	8	
Alcohol current use, *N* (%)				
. No	46 (24%)	25 (18%)	18 (49%)	<.001

. Weekly	19	(10%)	7	(5%)	9	(24%)	
. Daily	124	(66%)	108	(77%)	10	(27%)	
. Unknown	75		56		16		
Localisation, *N* (%)							
. Soft palate	12	(5%)	11	(6%)	0	(0%)	0.002
. Tonsil	114	(46%)	72	(40%)	33	(66%)	
. BOT/vallecula**	88	(36%)	67	(37%)	16	(32%)	
. Pharyngeal wall	31	(13%)	30	(17%)	1	(2%)	
. Unclear	19		16		3		
Concurrent treatment, *N* (%)							
. None	100	(39%)	78	(40%)	16	(32%)	0.53
. Chemo	152	(58%)	111	(57%)	32	(64%)	
. EGFR inhibition	7	(3%)	5	(3%)	2	(4%)	
. Censored (no radiotherapy or missing information)	5		2		3		
OTT, Median (Q1, Q3)	44.0	(42.0, 47.0)	44.0	(42.0,47.0)	44.0	(42.0,48.0)	0.45

Differences between HPV- and HPV+ groups were analyzed using the Chi square test for gender, tonsillar localization, concurrent treatment. The Mann-Whitney U test was used for age, UICC stage number, pack years, alcohol use, OTT.

** For some patients HPV status could not be determined. The characteristics of this group are not shown. Therefore the sum of the HPV positive and negative subgroup is equal or lower that the number shown for the whole patient population. ** BOT, base of tongue. $UICC 7th edition*

3.2. Incidence trends of Head and neck carcinoma and oropharyngeal carcinoma in Flanders: from 1999 to 2010.

From the Belgian Cancer Registry (www.kankerregister.org) the crude incidence rates for males and females between 1999 and 2010 were obtained for head and neck cancer in general and more specific for the oropharyngeal subsite to investigate incidence trends over time. As shown in figure 16, the incidence of head and neck cancer in general remains stable whereas the incidence rate (N/100.000) for oropharyngeal cancers increases significantly (2.1% each year) in males. In females the trend is somewhat different. We note an overall increase in head and neck cancer of 3% yearly but only a 1.2% yearly increase for oropharyngeal cancers.

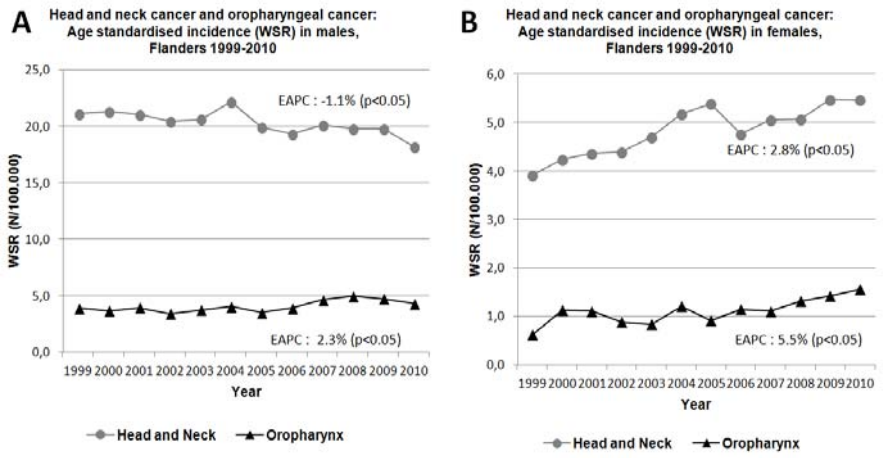


Figure 16: ***Incidence rates of Head and Neck cancer in total and cancers of the oropharyngeal subsite according to gender****. Data are based on crude incidence rates (N/100.000) for the Flemish region, obtained from the National Cancer Registry (Belgium). EAPC, Estimated Annual Percentage of Change. The p-value indicates if there is a significant change over time*

3.3. Prevalence of HPV related oropharyngeal disease

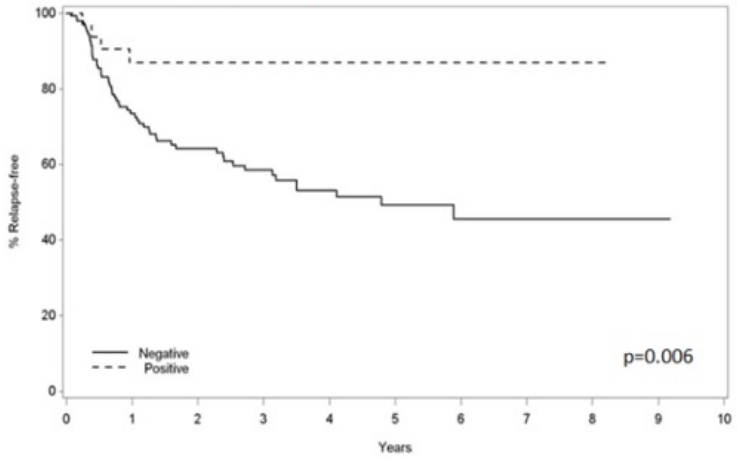
For a small number (*15* cases, see table 3) of observations there was missing information on HPV status. In *2* of *15* cases there was no information on p16 IHC. In

the remainder of the cases there was positive p16 IHC, but no PCR information on HPV status (e.g. insufficient amount or insufficient quality of extracted DNA). Patients with negative p16 IHC were regarded as HPV(-) [165]. In case of random missingness (equal probability of unknown HPV status for HPV- and HPV+) , missing information would not affect the estimate of the prevalence. However, as a result of the applied algorithm, we observed that all cases with unknown HPV status but known p16 status had positive p16 status. This suggested that the probability of unknown HPV status is larger for HPV(+) compared to HPV(-), and therefore that the prevalence of HPV may be underestimated if we only use the observed data for prevalence estimation. To account for this we provide prevalence estimates accounting for missing data by means of Inverse Probability Weighting (IPW). Based on this analysis the estimated prevalence of HPV related squamous cell carcinoma located in the oropharyngeal subsite is 24.78% (19.93-30.36%). Additionally we observed that HPV+ cases are significantly ($p<0.05$) associated with higher UICC tumor stage, lower alcohol and tobacco usage, and a tonsillar or base of tongue sublocalization in the oropharynx (Table 3). Of note, in our study population the HPV+ patients had a significantly elder age at diagnosis (62 vs. 58 years, $p=0.028$). The prevalence of HPV(+) disease was similar in males as in females.

3.4. Prognostic effect of HPV status after ablative radiotherapy

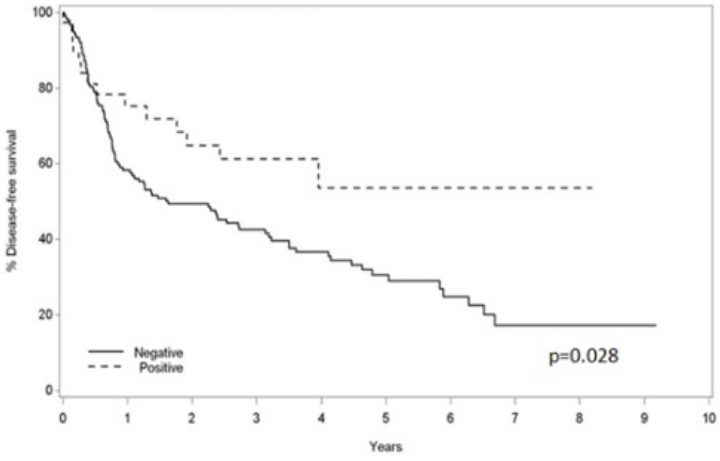
In this analysis only patients who received ablative dose radiotherapy ($\geq$60Gy) on macroscopic tumor were included. Positive HPV status was associated with a better locoregional control (LRC) after radiotherapy at 1 and 5 year (87% and 87%) when compared to the HPV(-) group (73% and 49%, $p=0.006$; Fig. 17A). The Disease Free survival (DFS) was also better in the HPV (+) group (75% and 54%) in comparison to the HPV(-) patient group (58% and 31%) at 1 and 5 years ($p=0.028$; Fig. 17B). Further for overall survival (OS) we noted a trend for better outcome in the HPV positive group where the OS was 84% at 1 year and 45% at 5 years in comparison to 67% and 38% in the HPV negative group ($p=0.09$ Fig. 17C). Finally we estimated the effect of

A **Kaplan-Meier curve for locoregional tumor control: HPV- and HPV+**



Number at risk											
Negative	154	82	65	47	35	19	12	4	2	1	0
Positive	38	23	18	12	7	6	4	3	2	1	1

B **Kaplan-Meier curve for disease-free survival: HPV- and HPV+**



Number at risk											
Negative	153	79	64	47	35	19	12	4	2	1	0
Positive	38	23	18	12	7	6	4	3	2	1	1

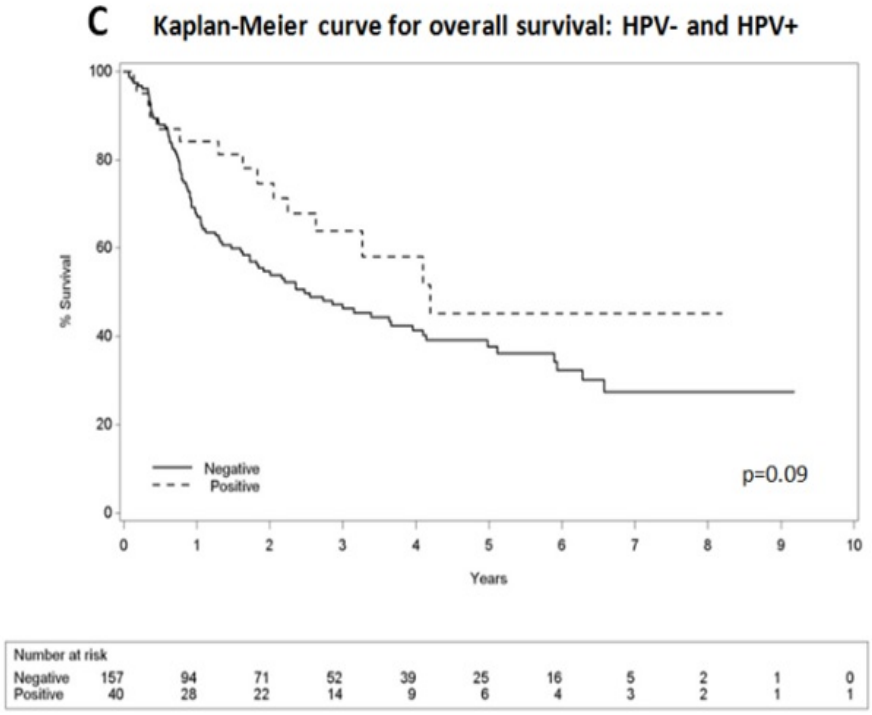


Figure 17: (A-C): ***Kaplan Meier estimates for survival among patients with an oropharyngeal carcinoma treated with ablative doses of radiotherapy (≥60Gy) on macroscopic tumor stratified according to HPV-status.*** *Figures show Locoregional tumor Control (A, page to the left), Disease Free Survival (B, page to the left) and Overall Survival (C, above). Numbers at risk at each stage of follow-up are shown in a box below each specific graph.*

HPV status on outcome, correcting for possible confounders significantly associated with HPV status in a Cox proportional hazards model. Included confounders were tumor stage, age, smoking, drinking and tumor localization (see Table 3). Since there was no association between concurrent chemotherapy or overall treatment time and HPV-status, they were not included as possible confounders. After correction of the higher described confounders positive HPV status remained significantly associated

with better LRC (Hazard Rate (HR) 0.21, *p=0.02*). The associations between HPV status and DFS/OS were not significant in multivariate analysis.

3.5 The prognostic effect of smoking and concurrent chemotherapy in HPV(+) oropharyngeal cancer.

Smoking and concurrent chemotherapy are well known parameters affecting the outcome in classical HPV negative head and neck cancer. Whether these parameters are equally important in HPV positive head and neck cancer is not as clear.

To investigate the effect of smoking as an independent predictor of outcome next to HPV status, a multivariate model was built including both smoking and HPV status as predictors. In our series we found a significantly better DFS for never smokers as compared to smokers (HR 0.39, *p=0.036*) but not when compared to former smokers. Regarding OS, we also noted a clear trend towards better outcome for never smokers compared to current smokers (HR 0.45, *p=0.072*). Smoking seemed not to be an independent risk factor predicting LRC.

To answer the question if concurrent chemotherapy is associated with better LRC and OS in HPV(+) patients, the interaction between HPV status and concurrent chemotherapy was tested and was found to be non-existing. Therefore, we also tested for the main effect of concurrent chemotherapy, which is than assumed to be equal in HPV(-) and HPV (+) patients. A significantly better LRC was found for concurrent chemoradiotherapy (HR= 0.59, *p=0.0462*) was found. We did not find a significant association towards OS.

4. Conclusion and discussion

HPV-associated oropharyngeal cancer is currently an emerging global pandemic. This new viral etiology is held responsible for the current rise in incidence registered by the American SEER database by 2-3% each year. However large geographical

differences in prevalence rates seem to exist [12,95]. For the region of Flanders no data are available.

Data obtained from the National Cancer Registry of Belgium however demonstrated comparable to American series a yearly increase of 2.1% in the crude incidence rates of oropharyngeal carcinoma for males (Fig. 16A) in the region of Flanders. In contrast, the general incidence of head and neck cancer seemed to remain stable, suggesting that this rise was not related to increased exposure of the patient population to classical risk factors like tobacco and alcohol exposure. If so, they would increase the incidence of head and neck cancer in general as well. In females however an increase in oropharygeal carcinoma incidence was noted, but it was also accompanied by a raise in head and neck cancer in general as well, making a possible contribution of HPV less clear.

In our series, a multicentre collaboration between the different radiation-oncology departments of the Flemish universities, we document a prevalence of HPV induced oropharyngeal carcinoma of 24.78% (19.93-30.36%) using a well validated algorithm making use of p16 immunohistochemistry and HPV-PCR [165]. These data are comparable to the prevalences reported in our neighboring regions as the Netherlands (23.3-29%), France (17.2%) and Germany (16.7-62.9%) [12,166], although some of these series may not represent the actual prevalence rates anymore because of their earlier date. The relative contribution of HPV related disease in male and female subgroups was similar.

HPV related cancers were found to be associated with higher tumor stage, lower alcohol or tobacco exposure, and tonsillar of base of tongue sublocalization (Table 3) in our series. Also survival analysis showed a significant improvement for LRC, DFS and OS of 85% vs. 50%, 54% vs. 30% and 47% vs. 38% in favor of HPV related pathology at 5 years (Fig. 17A-C). After multivariate analysis HPV remained a strong independent factor associated with better locoregional control after radiotherapy (HR=3.74, *p=0.02*). These findings are all in agreement with other published series

[9,10,13,15,16,111,173]. On the other hand, in contrast to many other series we found HPV(+) status in Flemish patients to be associated with elder rather than younger age.

Recent studies suggest both smoking and HPV status are independent prognostic factors regarding outcome for patients with oropharyngeal carcinoma treated with radiotherapy [15,174]. In our series we could not confirm or disprove these observations although regarding disease free survival we did see a trend (p=0.064) approaching statistical significance for smoking status with HR's of stopped smokers and current smokers of 1.64 and 2.49 respectively versus non-smokers.

Concurrent radio-chemotherapy is a very toxic treatment but has been showing consistently improved outcome in classical head and neck cancer [35,175]. However a more recent report claimed that in advanced stage HPV associated oropharyngeal carcinoma patients receiving radiotherapy alone had similar locoregional control rates as patients receiving concurrent radiochemotherapy [176] which was a interesting observation regarding it as a possible treatment deintensification strategy. In our series however, we did not find any evidence for a difference in effectiveness for concurrent chemoradiotherapy between HPV(+) and HPV(-) patients and noted a clear trend towards improvement of locoregional control with the addition of chemotherapy (HR = 0.61*, p=0.056*) for both subgroups.

CHAPTER VIII

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. FLT1 as a new target in HNSCC

The FLT1/VEGFR1 receptor is primarily known as a negative regulator in angiogenesis, where its primary function lies in scavenging VEGFA by its extracellular domain, and thereby preventing VEGFA binding and activation of KDR/VEGFR2 [150]. Recently however, FLT1 has also been reported to be an oncogenic driver in tumor types like breast, colon, and skin cancer [154-156]. Here, by making use of an unbiased systematic screening approach towards the whole tyrosine kinase family, we documented and validated FLT1 to be important for the survival and radioresistance of HNSCC cells.

The main aim of this doctoral thesis was to establish new therapeutic targeting strategies based on essential tyrosine kinase signal transduction in head and neck squamous cell carcinoma cell lines, in a translational research project. We tried to uncover key nodes in the tyrosine kinase machinery responsible for resistance to radiotherapy (and cell survival) and investigate the clinical potential of specific targeting strategies towards these nodes, rather than to uncover the precise molecular pathways and molecular mechanisms leading the increased radioresistance. Therefore, let us benchmark the results obtained in this thesis towards the earlier described 'seven essential characteristics of a good candidate for targeted therapy' (Chapter I, 2.2) in this context.

The first essential characteristic was the expression of the target molecule in the cancer cells. In this light we did show that FLT1 is indeed ubiquitously expressed in HNSCC. Experiments on cell lines indeed confirmed FLT1 protein expression in all of our HNSCC cell lines (data not shown). Additional IHC experiments further confirmed the presence of this kinase in the vast majority of primary head and neck tumors (136/151, 90%). Because the number of tumors eventually not showing FLT1 expression is very low, it is possible that the negative staining in these few tumors may result from technical problems associated with the IHC technique, rather than FLT1 expression being actually absent. Such parameters can be the time and nature

of tissue fixation, the methods used to process tissue, or the temperature of paraffin embedding, as well as the storage time of unstained slices. These lessons were well learned from the IHC detection of Her2/C-erb2 protein overexpression in breast cancer [177-179]. However, the expression level a therapeutic target protein does not necessarily correlate with its validity as an oncogenic driver. A very nice example of this are earlier clinical studies investigating the therapeutic potential of EGFR-targeting cetuximab in colorectal cancer. Here there seemed no correlation between EGFR-expression level and response to therapy: even dramatic clinical responses were seen in EGFR low/negative tumors [180,181]. Somewhat in line with these observations we report no relation between local control after radiotherapy (a surrogate for *in vivo* radioresistance) in oropharyngeal HNSCC and the level of FLT1 expression. Moreover, our HPV associated HNSCC cell line SCC090, with proven FLT1 expression but low autocrine VEGFA production does not show a decrease in survival after sunitinib mediated FLT1 inhibition, meaning that the expression of FLT1 does not implicate its role as an oncogenic driver in HNSCC.

In the context of a newly identified oncogene like FLT1, another essential characteristic of a good candidate for targeted therapy is protein activation. For tyrosine kinases, tyrosine phosphorylation –a property which we did confirm in our HNSCC cell lines for FLT1- is general accepted as a good parameter to assess its activation [85]. Additional indirect proof comes from inhibitor experiments where FLT1 kinase inhibition shows similar effects on radioresistance and survival as siRNA and shRNA mediated FLT1 knockdown *in vitro*. Direct proof for *in vivo* receptor activation is however lacking in this work. Nonetheless, we show autocrine production of FLT1 activating ligands in HNSCC, *in vivo* as well as *in vitro*. Additionally, we learned from our *in vitro* experiments that the level of VEGFA activating ligand expression correlates to FLT1 mediated functions like radioresistance and decreased cell survival following FLT1 inhibition.

We have shown many different experiments demonstrating that FLT1 is indeed important towards HNSCC survival and radiotherapy resistance. We did this in independent HNSCC cell lines using different inhibitors, siRNA and shRNA, proving indeed the phenomenon of FLT1 oncogene addiction in HNSCC *in vitro* at least in a subset of HNSCC. In vivo experiments are still lacking and surely need to be performed in the future. The lack of a good specific FLT1 inhibitor however hinders this approach at the moment. To date indeed, no good specific FLT1 inhibitor exists. In fact, the currently available candidates are mostly inhibitors designed as anti-angiogenic therapy against KDR with additional off-target anti-FLT1 activity. This is also the case for the inhibitors axitinib and sunitinib used in this study. Although some data are available for FLT1 inhibitor effectiveness from *in vitro* assays, it is currently not known if tumoral FLT1 can be inhibited using these inhibitors *in vivo* (see Chapter VIII, 2). Of course these non specific inhibitors could be used for in vivo testing. However, as they are expected to also have a profound effect on angiogenesis (through KDR inhibition) it would be difficult to credit a possible tumor regression or increased radiosensitivity to an inhibitory effect through FLT1. These effects can indeed also be explained by inhibition of angiogenesis [182-184]. An *in vivo* inducible FLT1 knockout model could however be able to provide the needed proof of concept of a possible efficacy of FLT1 targeted therapy. This approach would however not be clinically applicable. On the other hand, there are already *in vivo* data available showing FLT1's essentiality towards tumor growth in squamous cell carcinoma of the skin [155].

Experiments with FLT1-kinase-dead-mice have demonstrated that FLT1-kinase function seems completely expendable for normal life and development. Therefore this kinase seems to meet the requirement of being a tumor specific target [151]. However, to the best of our knowledge, nothing is known about the sensitivity of the normal tissues in these mice towards radiotherapy. If these FLT1-kinase-dead-mice would show a higher radiosensistivity of the normal tissues, this property would abolish or at least diminish the therapeutic potential of a combined radiotherapy and

FLT1-targeting approach in HNSCC. Therefore we believe this topic needs further study.

Our *in vitro* studies clearly showed proof of concept that FLT1 kinase is indeed a targetable oncogenic driver. As illustrated from our *in vitro* data using sunitinib on SCC090, not all HNSCC cell lines seem to be FLT1 driven. It is therefore important to be able to determine in advance if a HNSCC tumor will or will not respond to FLT1 targeting. This is where appropriate biomarker strategies come in. An adequate selection of patients whom you will expose to these kind of therapies is extremely important, as we have learned from the past: effective treatment strategies may indeed fail to show an improvement in outcome it not the correct group of patients is treated. A nice example of this is an analysis done by Simon and colleagues [185]. They demonstrated that trastuzumab (a highly effective therapy in metastastic HER2-overexpressing breast cancer) probably would have failed to show any survival benefit if the study would have been performed in unselected patients [185]. Another example is the story of gefitinib in non small cell lung cancer (NSCLC). Here only after 2 negative phase III trials [186,187], it was discovered that only EGFR mutated lung cancers would be responsive to EGFR targeted therapy [188]. And later it was indeed shown that gefitinib was detrimental to patients having EGFR wild type tumors but beneficial to patients showing EGFR mutated adenocarcinoma of the lung [189]. An adequate biomarker will select the right patients for a certain targeted therapy and will also prevent other patients from drug exposure related toxicity if no benefit is expected. Besides, an adequate patient selection strategy will also make clinical trials more cost-effective.

In this context, we did very interesting observations regarding possible predictive biomarkers for FLT1 targeted therapy. We observed that FLT1 targeting using TKI's or siRNA seems to have a stronger radiosensitizing effect in our cell line with the higher VEGFA expression. We also observed failure of FLT1 targeted therapy using sunitinib in SCC090, our HNSCC cell line with the lowest VEGFA expression. Further over a panel of different cell lines derived from primary HNSCC, we document a strong

correlation between autocrine VEGFA expression and radioresistance, a property which we showed to be FLT1 dependent. In accordance with these observations, it has been known that higher VEGFA expression predicts for worse local control in primary HNSCC after radiotherapy [71,72]. Taken together, it is likely that the level of expression of the FLT1 activating ligand VEGFA correlates with FLT1 receptor activation and therefore would also identify the HNSCC tumors with a) activated FLT1, b) subsequent FLT1- oncogene addiction resulting in c) increased radioresistance. If the response towards FLT1 targeting is dependent on HPV status in HNSCC, as might be suggested by our observations using sunitinib on our HPV positive and negative cell lines (SCC090, SCC61 and SQD9), needs further investigation.

The final essential characteristic of a good candidate for targeted therapy is that of numerical importance. Our observations, showing VEGFA expression as well as FLT1 expression in nearly all HNSCC biopsies and cell lines, suggest this is indeed the case for FLT1. However, as we notice that within our panel of HNSCC cells both the response towards FLT1 targeted therapy and the radioresistance correlate closely to the highly variable VEGFA expression levels, we believe that the relative importance of FLT1 as an oncogenic driver within a specific HNSCC cell line is probably a continuum: on the one hand we have cells with very low VEGFA expression and subsequently no relevant FLT1 activation resulting in no impact of FLT1 targeting strategies. On the other hand we have cells with high VEGFA expression levels, showing FLT1 activation and oncogene addiction. Currently we have no idea about what level of VEGFA expression reflects a relevant contribution of FLT1 towards radioresistance and tumor growth in the context of FLT1 targeted therapy. We believe more insight may be obtained from examining several patient derived HNSCC xenograft models using a clinically applicable FLT1 targeting strategy.

2. FLT1 Targeting strategies: problems and opportunities

As demonstrated in Chapter V, plasma membrane permeability is an essential characteristic for a FLT1 inhibitor in the context of HNSCC because of the intracellular localization of FLT1 in this disease. However another (and more problematic) characteristic of an FLT1 targeting drug is the ability to interfere with FLT1 signaling at exposure levels achievable *in vivo*. As can be seen from our survival assays using axitinib and sunitinib performed with our HNSCC cell lines, the inhibitory effect on cell growth or survival kicks in only at concentrations of 0.5 µM or above. The achievable plasma concentrations of sunitinib and axitinib reported by the Food and Drug Administration (FDA) are 0.254 µM and 0.071 µM respectively. Therefore it is expected that FLT1 signaling can't be efficiently altered using these drugs in a patient oriented setting. This was one of the reasons why no *in vivo* experiments were undertaken to test the effect of these inhibitors: the outcomes of these experiments would not be clinically relevant. As discussed earlier, the currently available FLT1 targeting TKI's are inhibitors designed as anti-angiogenic therapy targeting predominantly KDR with 'off-target' anti-FLT1 activity. This KDR targeting activity is problematic, first of all because an additional profound effect on angiogenesis through KDR may result in unpredictable outcome: A well balanced angiogenic effect of such a compound may result in vascular normalization and increased radiosensitivity through better oxygen supply. However on the other hand a somewhat stronger anti-angiogenic effect may result in hypo-vascularisation, where lack of oxygen supply results in increased radioresistance. The use of the currently available FLT1 TKI's therefore adds an additional layer of complexity toward the predictability of treatment outcome which in my opinion is better to be avoided. Furthermore, additional KDR inhibitory activity will also result in additional toxicity. A more specific and more powerful FLT1 TKI is therefore awaited.

In this context, another compound of special interest is Farnesiferol C, a herbal compound which seems to interfere with VEGFA-FLT1 but not VEGFA-KDR signal

transduction [190]. This compound seems to be well tolerated. Moreover, Farnesiferol C is a coumarin, a class of drugs which is characterized by good plasma membrane permeability [191]. Unfortunately we were not able to obtain this compound for testing on our HNSCC cells.

Targeting strategies based on peptides or monoclonal antibodies seem problematic, because of their poor membrane permeability resulting from their greater size and lower lipophilicity. Although multiple drug-delivery strategies exist (e.g. cell penetrating peptide sequences, nanoparticles) [192,193], the delivery of these compounds to the correct cellular compartments remains a daunting problem [192]. Furthermore, it is currently not known in which subcellular compartment FLT1 is activated, meaning this topic requires further study.

Finally there is also the possibility to target FLT1 *expression* in HNSCC. A strategy using DNA antisense therapy to target tyrosine kinase expression in HNSCC has already shown promising results indeed towards EGFR [194]. The application was both safe and effective, although it required direct injection of the antisense DNA into the tumoral lesion. This of course impedes routinely clinical application. Another interesting setup is the use of shRNA molecules coupled to an RNA-based aptamer to ensure tumor specific uptake. Interesting preclinical results have already been obtained in l *in vivo* prostate cancer models to silence DNA repair proteins [195]. SiRNA, shRNA or antisense-DNA can also be delivered using nanoparticles [196]. The challenge in these approaches is to deliver these nuclear acids specifically within tumor cells. However, although it has been shown that FLT1 kinase function seems expendable for normal life, total FLT1 loss is not [151,197]. A non-tumor-specific FLT1 knockdown strategy may therefore result is significant toxicity, related to vascular overgrowth.

3. FLT1 from bench to bedside: many milestones are still ahead

We identified FLT1 as a new target to modulate the survival and radiosensitivity of HNSCC cells with high clinical potential (see Chapter VIII, 1). However several key scientific questions first need to be answered before FLT1 targeted therapy can be brought to clinical practice. First of all, the role of FLT1 in HNSCC survival and especially radioresistance needs to be confirmed *in vivo*. An inducible shRNA mediated FLT1 knockdown model may be used to verify this matter. Secondly, as discussed earlier, verifying if FLT1-TK knockout models do or do not experience increased radiosensitivity [151] of the normal tissues will be an important go/no-go experiment towards the development of FLT1 targeted therapy in HNSCC oncology. Thirdly, a better FLT1 inhibitor is needed (see discussion Chapter VIII, 2), showing good FLT1 inactivating ability under drug exposure levels which are realistic *in vivo*. Finally, more thorough testing of potential biomarkers towards response prediction for FLT1 targeted therapy needs to be done. The use of several, well characterized patient derived xenograft models may provide us the information needed. In this context, experiments done by our group already point out that assays with the potential to assess intratumoral VEGFA expression in a (semi)quantitative way (like IHC, qPCR, Elisa,…) are of special interest. HPV status may also be of importance. Only with these additional questions answered, clinical testing of a FLT1 targeted strategy may be taken towards Phase I-III clinical testing with confidence.

4. Tyrosine kinases affecting HNSCC survival but not radiosensitivity

Next to the tyrosine kinases FLT1, (and the well established HNSCC target EGFR) also a handful of other kinases affecting HNSCC survival only was retained. Since the aim of this thesis was to define new starting points to improve mainly the radiotherapy response in HNSCC these kinases were not further investigated, due to lack of time and resources. However due to a profound impact on cell survival these kinases could still be meaningful new targets. These kinases include BMX, AXL, TNK and TXK. A recent report linked AXL signal transduction to HNSCC invasion and migration, and

demonstrated that AXL overexpression correlates with tumor progression and poor prognosis [198]. BMX is mainly expressed in epithelial cells and has already been identified as an oncogenic driver in prostate cancer, bladder cancer and glioblastoma [199-201]. As BMX is a component of the PI3K pathway it is interesting to find BMX as a target in our 'PI3K mutated' cell line SCC61 [202].

5. New radiobiologic insight towards HPV associated HNSCC tumors

In this work it was attempted to shed light on the radiobiology of HPV-associated HNSCC tumors. The starting point of the study was the observation that among our HNSCC cell lines the lowest VEGFA expression correlating with the highest radiosensitivity was seen in our HPV(+) HNSCC lines (Chapter IV, Fig. 8). A diminished VEGFA expression associated with HPV(+) status was also seen on tumor biopsies derived from primary HNSCC (Chapter VI, Fig. 11). This lower VEGFA expression also correlated with a lack of response towards FLT1 targeted therapy in HNSCC cells (Chapter VI, Fig. 11) suggesting that HPV (+) HNSCC's might owe their decreased radioresistance to a lack of FLT1 activity. To study the nature of this decreased VEGFA expression we decided to study a possible role of p16 as a suppressor of the HIF1α pathway in HPV associated HNSCC. This was an interesting offset since a suppressed HIF1α pathway in HPV(+) HNSCC would not only explain the origin of the decreased VEGFA expression but it would also shed additional light on the biological mechanisms explaining their increased radiosensitivity, the lack of negative prognostic impact of hypoxia as well as the failure of hypoxia targeted therapy (see Chapter I, 4.2.2.5).

Although we clearly demonstrated p16 related HIF1α suppressive activity in HPV(+) HNSCC, we also demonstrated that this suppressive activity did not suffice to prevent a glycolytic shift in these cells. Possibly this is due to HIF1α stimulatory activity of the HPV oncoproteins [169]. We also could not show evidence supporting the hypothesis of p16 compromising survival of HPV(+) HNSCC cells under hypoxia, a process that would (partly) explain why HPV related tumors are more radiocurable and why

hypoxia targeted drugs seem ineffective in this setting. Nonetheless, since hypoxic conditions can be more pronounced *in vivo* than in our *in vitro* conditions (1% O_2), this hypothesis cannot be completely rejected by our experimental findings. The reason why HPV(+) HNSCC is not responsive on hypoxia targeted therapy therefore remains obscure, although new insight came from a very recent publication by Sorensen et al [203]. Next to showing that there is no difference between the hypoxic gene response between HPV(+) and (-) HNSCC cells in anoxic (0% O_2) and normoxic conditions (21% O_2) they also showed that the hypoxia sensitizing effect of nimorazole was comparable in both types of cells. The authors suggested therefore that the failure of nimorazole to sensitize HPV positive cells *in vivo* might result from 'overkill' since HPV(+) cells seemed more radiosensitive. This means that as radiotherapy may be already inducing sufficient cell kill as a monotherapy, there would be no benefit of intensifying the treatment as it is only possible to kill all cells once. Nonetheless, based on these data it is also very reasonable to assume that the explanation may be related to processes in the microenvironment such as tumor vasculature or the immune system. A decreased VEGFA expression in response to radiotherapy may indeed fail to protect the tumor vasculature from radiotherapy in HPV positive cells for example [204]. The result could then be a more pronounced vascular disruption and increased tumor kill. On the other hand, as radiotherapy co-acts with the immune system to eradicate tumor cells [127], it is possible that the unique interaction between the viral oncogenes, the immune system and hypoxia may explain these findings. For example it is possible that improved immune response, resulting from hypoxia and viral antigen mediated stimulation of antigen presenting cells [205,206] selectively compensates for the detrimental effect of hypoxia in HPV(+) cells only.

6. The epidemiology of HPV related oropharyngeal cancer in Flanders

As already discussed, a subgroup of HNSCC is etiologically related to Human Papilloma Virus (HPV) infection. Recently it became clear that this is a different tumor

entity, with distinct clinical behavior, affecting younger people with higher socio-economical status. These cancers are predominantly originating in the oropharynx. According to American SEER data, this subgroup is becoming a new epidemic, with its incidence increasing 2-3% each year. American studies have shown that 40 to 65% of HNSCC arising from the oropharynx could be attributed to HPV16 [10,11,12,13]. It has also been shown that these tumors respond far better to radiotherapy and chemotherapy treatments [15,16]. However, as already mentioned they do not respond to hypoxia targeted treatment [207,16]. These factors could probably have an impact on treatment selection. Currently, locally advanced stage oropharyngeal cancers are being treated in the same way as the classical HNSCC with radio-chemotherapy schedules. These intense treatment regimens come however at the cost of severe acute and late toxicity (like dysphagia and feeding-tube dependency) [35]. Patients with HPV-associated HNSCC might not need these intense treatment regimens, and perhaps more function sparing therapies lead to equal survival numbers in this population. To set up new studies, we need to have an idea about the prevalence of HPV in oropharyngeal cancer in our patient population. However, large geographical differences seem to exist. In the United States 40 to 80% of oropharyngeal tumors are associated with HPV, while in Europe the proportion varies from 90% in Sweden to about 20% in communities with high tobacco exposure [95]. Before our study was undertaken, no data were available for Flanders nor Belgium.

The technical aspects of HPV testing may affect prevalence numbers and may be partly responsible for the large geographical differences seen in Europe, as for example PCR techniques are highly susceptible to DNA contamination between unrelated samples [121,122]. Therefore HPV testing was performed with extreme care using a validated approach making use of double testing. We used a GP5+6+ primer pair based PCR to detect HPV DNA together with p16 IHC, an approach showing an accuracy for HPV testing on paraffin embedded tumor blocks approaching 100% [165,166]. To avoid false negatives the amplifiability of the extracted DNA was tested, and the presence of tumor tissue in the extracted tissue was verified using the

sandwich method. Further the used PCR test was suited to detect a very wide range of high-risk HPV subtypes, in contrast to several in situ hybridization probes. Next to p16 immunohistochemistry, we also changed microtome blades between each microtome cut tissue block to avoid false positives. We are therefore very confident that the found prevalence level of HPV related oropharyngeal cancers are accurate and reliable.

The prevalence of HPV(+) oropharyngeal cancer in Flanders was found to be 24.78% (19.93-30.36%). Further we were also able to show that the incidence of oropharyngeal carcinoma in Flanders is on the rise in both males (2.3% yearly) and females (5.5% yearly). These data are essential for writing new clinical study protocols investigating more organ spearing approaches for these HPV-positive HNSCC patients with better prognosis. These data may become relevant for clinical policy making towards treatment follow-up and prevention campaigns for example.

Our study confirms data from other series by showing HPV(+) tumors correlated with more advanced tumor stage, less smoking and alcohol consumption, tonsillar/base of tongue sublocalization and better outcome after radio(chemo)therapy. Interestingly, we saw that HPV(+) tumors were associated with elder age in Flanders. The reason for this in not clear but it is possible that differences in oral sex practice between Flanders and the United States exist.

The younger age and improved survival of HPV-positive patients implies that a reduction of treatment related toxicity may be necessary and feasible. In this context, a more recent report claimed that in advanced stage HPV associated oropharyngeal carcinoma radiotherapy alone had similar LRC rates compared to concurrent radiochemotherapy. In our series however, we did not find any evidence for a difference in effectiveness for concurrent chemoradiotherapy between HPV(+) and HPV(-) patients. Our data are in line with the data obtained from (HPV associated) cervix carcinoma, where the addition of chemotherapy results in better outcome [208]. Therefore we believe that treatment de-escalation by omitting concurrent

chemotherapy is probably not a good idea. If the more toxic chemotherapeutic cisplatin can be replaced by the less toxic EGFR directed monoclonal antibody cetuximab is currently under investigation in the 'RTOG-1016 and 'De-ESCALaTE HPV' randomized clinical trials.

As HPV positive tumors are more radiosensitive [15,16,111] another possible strategy to reduce treatment related toxicity is the reduction of the standard dose of definitive radiotherapy. An interesting approach where HPV positive tumors with a complete remission after induction chemotherapy receive a reduced dose of 54 Gy is currently under investigation by the Eastern Cooperative Oncology Group (ECOG) in a phase II trial.

References

1 Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. Lancet 2008;371:1695-709.

2 Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011;11:9-22.

3 GLOBOCAN 2008 (IARC).

Section of Cancer Information. Last updated on 25/8/2013. Retrieved from: http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900 on 25/08/2013. 2013;

4 Marur S, Forastiere AA. Head and neck cancer: changing epidemiology, diagnosis, and treatment. Mayo Clin Proc 2008;83:489-501.

5 Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003;3:733-44.

6 Andre K, Schraub S, Mercier M, Bontemps P. Role of alcohol and tobacco in the aetiology of head and neck cancer: a case-control study in the Doubs region of France. Eur J Cancer B Oral Oncol 1995;31B:301-9.

7 Murata M, Takayama K, Choi BC, Pak AW. A nested case-control study on alcohol drinking, tobacco smoking, and cancer. Cancer Detect Prev 1996;20:557-65.

8 Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. Nat Rev Cancer 2007;7:599-612.

9 Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20.

10 Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008;100:261-9.

11 Gillison ML, Lowy DR. A causal role for human papillomavirus in head and neck cancer. Lancet 2004;363:1488-9.

12 Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 2005;14:467-75.

13 Kumar B, Cordell KG, Lee JS, Worden FP, Prince ME, Tran HH et al. EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. J Clin Oncol 2008;26:3128-37.

14 Sturgis EM, Cinciripini PM. Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer 2007;110:1429-35.

15 Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010;363:24-35.

16 Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol 2010;28:4142-8.

17 Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006;367:1747-57.

18 Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med 2001;345:1890-900.

19 World Health Organisation Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. Lyon: IARC Press 2005

20 Gregoire V, Lefebvre JL, Licitra L, Felip E. Squamous cell carcinoma of the head and neck: EHNS-ESMO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010;21 Suppl 5:v184-v186.

21 Nilssen EL, Murthy P, McClymont L, Denholm S. Radiological staging of the chest and abdomen in head and neck squamous cell carcinoma--are computed tomography and ultrasound necessary? J Laryngol Otol 1999;113:152-4.

22 Brown DH, Leakos M. The value of a routine bone scan in a metastatic survey. J Otolaryngol 1998;27:187-9.

23 Yoo J, Henderson S, Walker-Dilks C. Evidence-based guideline recommendations on the use of positron emission tomography imaging in head and neck cancer. Clin Oncol (R Coll Radiol) 2013;25:e33-e66.

24 Joiner M, van der Kogel A. Basic Clinical Radiobiology, 4th edition. Hodder Arnold 2009;

25 Dirix P, Nuyts S. Evidence-based organ-sparing radiotherapy in head and neck cancer. Lancet Oncol 2010;11:85-91.

26 Vissink A, Jansma J, Spijkervet FK, Burlage FR, Coppes RP. Oral sequelae of head and neck radiotherapy. Crit Rev Oral Biol Med 2003;14:199-212.

27 Bernier J, Domenge C, Ozsahin M, Matuszewska K, Lefebvre JL, Greiner RH et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med 2004;350:1945-52.

28 Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med 2004;350:1937-44.

29 Vermorken JB, Remenar E, van HC, Gorlia T, Mesia R, Degardin M et al. Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. N Engl J Med 2007;357:1695-704.

30 Boscolo-Rizzo P, Gava A, Marchiori C, Baggio V, Da Mosto MC. Functional organ preservation in patients with locoregionally advanced head and neck squamous cell carcinoma treated by platinum-based multidrug induction chemotherapy and concurrent chemoradiotherapy. Ann Oncol 2011;22:1894-901.

31 Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003;349:2091-8.

32 Bourhis J, Overgaard J, Audry H, Ang KK, Saunders M, Bernier J et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. Lancet 2006;368:843-54.

33 Pignon JP, Bourhis J, Domenge C, Designe L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. Lancet 2000;355:949-55.

34 Trotti A. Toxicity in head and neck cancer: a review of trends and issues. Int J Radiat Oncol Biol Phys 2000;47:1-12.

35 Machtay M, Moughan J, Trotti A, Garden AS, Weber RS, Cooper JS et al. Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: an RTOG analysis. J Clin Oncol 2008;26:3582-9.

36 Bonner JA, Harari PM, Giralt J, Cohen RB, Jones CU, Sur RK et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol 2010;11:21-8.

37 Wu HC, Chang DC, Huang CT. Targeted Therapy for Cancer. Journal of Cancer Molecules 2006;2:57-66.

38 Weinstein IB, Joe A. Oncogene addiction. Cancer Res 2008;68:3077-80.

39 Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53:3579-84.

40 Hama T, Yuza Y, Saito Y, uchi J, Kondo S, Okabe M et al. Prognostic significance of epidermal growth factor receptor phosphorylation and mutation in head and neck squamous cell carcinoma. Oncologist 2009;14:900-8.

41 Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 2006;24:2666-72.

42 Ozanne B, Richards CS, Hendler F, Burns D, Gusterson B. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. J Pathol 1986;149:9-14.

43 Goessel G, Quante M, Hahn WC, Harada H, Heeg S, Suliman Y et al. Creating oral squamous cancer cells: a cellular model of oral-esophageal carcinogenesis. Proc Natl Acad Sci U S A 2005;102:15599-604.

44 Harari PM, Huang SM. Head and neck cancer as a clinical model for molecular targeting of therapy: combining EGFR blockade with radiation. Int J Radiat Oncol Biol Phys 2001;49:427-33.

45 Hoffmann T, Hafner D, Ballo H, Haas I, Bier H. Antitumor activity of anti-epidermal growth factor receptor monoclonal antibodies and cisplatin in ten human head and neck squamous cell carcinoma lines. Anticancer Res 1997;17:4419-25.

46 Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008;359:1116-27.

47 Loeffler-Ragg J, Witsch-Baumgartner M, Tzankov A, Hilbe W, Schwentner I, Sprinzl GM et al. Low incidence of mutations in EGFR kinase domain in Caucasian patients with head and neck squamous cell carcinoma. Eur J Cancer 2006;42:109-11.

48 Sheu JJ, Hua CH, Wan L, Lin YJ, Lai MT, Tseng HC et al. Functional genomic analysis identified epidermal growth factor receptor activation as the most common genetic event in oral squamous cell carcinoma. Cancer Res 2009;69:2568-76.

49 Franovic A, Gunaratnam L, Smith K, Robert I, Patten D, Lee S. Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc Natl Acad Sci U S A 2007;104:13092-7.

50 Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res 2006;12:5064-73.

51 Nyati MK, Morgan MA, Feng FY, Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer 2006;6:876-85.

52 Seiwert TY, Jagadeeswaran R, Faoro L, Janamanchi V, Nallasura V, El DM et al. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. Cancer Res 2009;69:3021-31.

53 Knowles LM, Stabile LP, Egloff AM, Rothstein ME, Thomas SM, Gubish CT et al. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. Clin Cancer Res 2009;15:3740-50.

54 Psyrri A, Seiwert TY, Jimeno A. Molecular pathways in head and neck cancer. Am Soc Clin Oncol Educ Book 2013;2013:246-55.

55 Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N. Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. Int J Oncol 2008;32:101-11.

56 Okami K, Wu L, Riggins G, Cairns P, Goggins M, Evron E et al. Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors. Cancer Res 1998;58:509-11.

57 Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y et al. Frequent Mutation of the PI3K Pathway in Head and Neck Cancer Defines Predictive Biomarkers. Cancer Discov 2013;3:761-9.

58 Qiu W, Schonleben F, Li X, Su GH. Disruption of transforming growth factor beta-Smad signaling pathway in head and neck squamous cell carcinoma as evidenced by mutations of SMAD2 and SMAD4. Cancer Lett 2007;245:163-70.

59 Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W et al. High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res 1996;56:3630-3.

60 Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J 2003;22:4212-22.

61 Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H et al. A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol Cell Biol 2002;22:5157-72.

62 Smeets SJ, van der Plas M, Schaaij-Visser TB, van Veen EA, van MJ, Braakhuis BJ et al. Immortalization of oral keratinocytes by functional inactivation of the p53 and pRb pathways. Int J Cancer 2011;128:1596-605.

63 Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 2011;333:1154-7.

64 Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011;333:1157-60.

65 Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet 2012;13:654-66.

66 Demarest RM, Dahmane N, Capobianco AJ. Notch is oncogenic dominant in T-cell acute lymphoblastic leukemia. Blood 2011;117:2901-9.

67 Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature 2011;475:101-5.

68 Klinakis A, Lobry C, Abdel-Wahab O, Oh P, Haeno H, Buonamici S et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature 2011;473:230-3.

69 Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van NM et al. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33:416-21.

70 Grothey A, Galanis E. Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol 2009;6:507-18.

71 Parikh RR, Yang Q, Haffty BG. Prognostic significance of vascular endothelial growth factor protein levels in T1-2 N0 laryngeal cancer treated with primary radiation therapy. Cancer 2007;109:566-73.

72 Smith BD, Smith GL, Carter D, Sasaki CT, Haffty BG. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. J Clin Oncol 2000;18:2046-52.

73 Tong M, Lloyd B, Pei P, Mallery SR. Human head and neck squamous cell carcinoma cells are both targets and effectors for the angiogenic cytokine, VEGF. J Cell Biochem 2008;105:1202-10.

74 Cohen EE, Rosen F, Stadler WM, Recant W, Stenson K, Huo D et al. Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol 2003;21:1980-7.

75 Fury MG, Sherman E, Lisa D, Agarwal N, Algazy K, Brockstein B et al. A randomized phase II study of cetuximab every 2 weeks at either 500 or 750 mg/m2 for patients with recurrent or metastatic head and neck squamous cell cancer. J Natl Compr Canc Netw 2012;10:1391-8.

76 Bauman JE, Michel LS, Chung CH. New promising molecular targets in head and neck squamous cell carcinoma. Curr Opin Oncol 2012;24:235-42.

77 Stegeman H, Kaanders JH, van der Kogel AJ, Iida M, Wheeler DL, Span PN et al. Predictive value of hypoxia, proliferation and tyrosine kinase receptors for EGFR-inhibition and radiotherapy sensitivity in head and neck cancer models. Radiother Oncol 2013;106:383-9.

78 Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol 2013;10:143-53.

79 Bussink J, van der Kogel AJ, Kaanders JH. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. Lancet Oncol 2008;9:288-96.

80 Herzog A, Bian Y, Vander BR, Hall B, Coupar J, Cheng H et al. PI3K/mTOR Inhibitor PF-04691502 Antitumor Activity Is Enhanced with Induction of Wild-Type TP53 in Human Xenograft and Murine Knockout Models of Head and Neck Cancer. Clin Cancer Res 2013;19:3808-19.

81 Roh JL, Kang SK, Minn I, Califano JA, Sidransky D, Koch WM. p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. Oral Oncol 2011;47:8-15.

82 Lehmann S, Bykov VJ, Ali D, Andren O, Cherif H, Tidefelt U et al. Targeting p53 in vivo: a first-in-human study with p53-targeting compound APR-246 in refractory hematologic malignancies and prostate cancer. J Clin Oncol 2012;30:3633-9.

83 Cuddihy AR, Bristow RG. The p53 protein family and radiation sensitivity: Yes or no? Cancer Metastasis Rev 2004;23:237-57.

84 Argiris A, Duffy AG, Kummar S, Simone NL, Arai Y, Kim SW et al. Early tumor progression associated with enhanced EGFR signaling with bortezomib, cetuximab, and radiotherapy for head and neck cancer. Clin Cancer Res 2011;17:5755-64.

85 Hunter T. Tyrosine phosphorylation: thirty years and counting. Curr Opin Cell Biol 2009;21:140-6.

86 Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene 2000;19:5548-57.

87 Hubbard SR. Structural analysis of receptor tyrosine kinases. Prog Biophys Mol Biol 1999;71:343-58.

88 Hubbard SR, Till JH. Protein tyrosine kinase structure and function. Annu Rev Biochem 2000;69:373-98.

89 Cooper JA, Bowen-Pope DF, Raines E, Ross R, Hunter T. Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. Cell 1982;31:263-73.

90 Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature 2001;411:355-65.

91 Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med 2005;353:172-87.

92 Slomiany MG, Black LA, Kibbey MM, Tingler MA, Day TA, Rosenzweig SA. Insulin-like growth factor-1 receptor and ligand targeting in head and neck squamous cell carcinoma. Cancer Lett 2007;248:269-79.

93 Zhu L, Werner JA, Mandic R. Implications of tropomyosin-related kinase B (TrkB) in head and neck cancer. Anticancer Res 2007;27:3121-6.

94 de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur HH. Classification of papillomaviruses. Virology 2004;324:17-27.

95 Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol 2010;11:781-9.

96 Szentirmay Z, Polus K, Tamas L, Szentkuti G, Kurcsics J, Csernak E et al. Human papillomavirus in head and neck cancer: molecular biology and clinicopathological correlations. Cancer Metastasis Rev 2005;24:19-34.

97 Fehrmann F, Klumpp DJ, Laimins LA. Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. J Virol 2003;77:2819-31.

98 Tomakidi P, Cheng H, Kohl A, Komposch G, Alonso A. Modulation of the epidermal growth factor receptor by the human papillomavirus type 16 E5 protein in raft cultures of human keratinocytes. Eur J Cell Biol 2000;79:407-12.

99 Schiller JT, Day PM, Kines RC. Current understanding of the mechanism of HPV infection. Gynecol Oncol 2010;118:S12-S17.

100 Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol 2006;208:152-64.

101 Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) 2006;110:525-41.

102 Matsukura T, Kanda T, Furuno A, Yoshikawa H, Kawana T, Yoshiike K. Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J Virol 1986;58:979-82.

103 Badulescu F, Crisan A, Badulescu A, Schenker M. Recent data about the role of human papillomavirus (HPV) in oncogenesis of head and neck cancer. Rom J Morphol Embryol 2010;51:437-40.

104 Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. Clin Cancer Res 2009;15:6758-62.

105 Li Y, Nichols MA, Shay JW, Xiong Y. Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb. Cancer Res 1994;54:6078-82.

106 Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 2008;100:407-20.

107 Posner MR, Lorch JH, Goloubeva O, Tan M, Schumaker LM, Sarlis NJ et al. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. Ann Oncol 2011;22:1071-7.

108 Hafkamp HC, Manni JJ, Haesevoets A, Voogd AC, Schepers M, Bot FJ et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. Int J Cancer 2008;122:2656-64.

109 Licitra L, Perrone F, Bossi P, Suardi S, Mariani L, Artusi R et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. J Clin Oncol 2006;24:5630-6.

110 Xu CC, Biron VL, Puttagunta L, Seikaly H. HPV Status and second primary tumours in Oropharyngeal Squamous Cell Carcinoma. J Otolaryngol Head Neck Surg 2013;42:36.

111 Lassen P. The role of Human papillomavirus in head and neck cancer and the impact on radiotherapy outcome. Radiother Oncol 2010;95:371-80.

112 DeWeese TL, Walsh JC, Dillehay LE, Kessis TD, Hedrick L, Cho KR et al. Human papillomavirus E6 and E7 oncoproteins alter cell cycle progression but not radiosensitivity of carcinoma cells treated with low-dose-rate radiation. Int J Radiat Oncol Biol Phys 1997;37:145-54.

113 Huang H, Li CY, Little JB. Abrogation of P53 function by transfection of HPV16 E6 gene does not enhance resistance of human tumour cells to ionizing radiation. Int J Radiat Biol 1996;70:151-60.

114 Kamradt MC, Mohideen N, Vaughan AT. RU486 increases radiosensitivity and restores apoptosis through modulation of HPV E6/E7 in dexamethasone-treated cervical carcinoma cells. Gynecol Oncol 2000;77:177-82.

115 Hampson L, El Hady ES, Moore JV, Kitchener H, Hampson IN. The HPV16 E6 and E7 proteins and the radiation resistance of cervical carcinoma. FASEB J 2001;15:1445-7.

116 Wang W, Sima N, Kong D, Luo A, Gao Q, Liao S et al. Selective targeting of HPV-16 E6/E7 in cervical cancer cells with a potent oncolytic adenovirus and its enhanced effect with radiotherapy in vitro and vivo. Cancer Lett 2010;291:67-75.

117 Zheng Y, Zhang J, Rao Z. Ribozyme targeting HPV16 E6E7 transcripts in cervical cancer cells suppresses cell growth and sensitizes cells to chemotherapy and radiotherapy. Cancer Biol Ther 2004;3:1129-34.

118 Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20.

119 Scholes AG, Liloglou T, Snijders PJ, Hart CA, Jones AS, Woolgar JA et al. p53 mutations in relation to human papillomavirus type 16 infection in squamous cell carcinomas of the head and neck. Int J Cancer 1997;71:796-9.

120 Sisk EA, Soltys SG, Zhu S, Fisher SG, Carey TE, Bradford CR. Human papillomavirus and p53 mutational status as prognostic factors in head and neck carcinoma. Head Neck 2002;24:841-9.

121 Snijders PJ, van den Brule AJ, Meijer CJ. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. J Pathol 2003;201:1-6.

122 van Houten VM, Snijders PJ, van den Brekel MW, Kummer JA, Meijer CJ, van LB et al. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. Int J Cancer 2001;93:232-5.

123 Lassen P. The role of Human papillomavirus in head and neck cancer and the impact on radiotherapy outcome. Radiother Oncol 2010;95:371-80.

124 Westra WH, Taube JM, Poeta ML, Begum S, Sidransky D, Koch WM. Inverse relationship between human papillomavirus-16 infection and disruptive p53 gene mutations in squamous cell carcinoma of the head and neck. Clin Cancer Res 2008;14:366-9.

125 Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med 2007;357:2552-61.

126 Kimple RJ, Smith MA, Blitzer GC, Torres AD, Martin JA, Yang RZ et al. Enhanced Radiation Sensitivity in HPV-Positive Head and Neck Cancer. Cancer Res 2013;73:4791-800.

127 Vu HL, Sikora AG, Fu S, Kao J. HPV-induced oropharyngeal cancer, immune response and response to therapy. Cancer Lett 2010;288:149-55.

128 Spanos WC, Nowicki P, Lee DW, Hoover A, Hostager B, Gupta A et al. Immune response during therapy with cisplatin or radiation for human papillomavirus-related head and neck cancer. Arch Otolaryngol Head Neck Surg 2009;135:1137-46.

129 Nagel R, Martens-de Kemp SR, Buijze M, Jacobs G, Braakhuis BJ, Brakenhoff RH. Treatment response of HPV-positive and HPV-negative head and neck squamous cell carcinoma cell lines. Oral Oncol 2013;49:560-6.

130 Dok R, Kalev P, Van Limbergen EJ, Vasquez I, Hauben E, Sablina A et al. p16/INK4A impairs homologous recombination-mediated DNA repair in human papillomavirus (HPV) positive-head and neck tumors. Cancer Res, submitted 2013;

131 Gubanova E, Brown B, Ivanov SV, Helleday T, Mills GB, Yarbrough WG et al. Downregulation of SMG-1 in HPV-positive head and neck squamous cell carcinoma due to promoter hypermethylation correlates with improved survival. Clin Cancer Res 2012;18:1257-67.

132 Rieckmann T, Tribius S, Grob TJ, Meyer F, Busch CJ, Petersen C et al. HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. Radiother Oncol 2013;107:242-6.

133 Cha Y, Issaeva N, Yarbourgh WG. Sensitivity of HPV (+) Oropharyngeal Head and Neck Cancers to Poly(ADP-ribose) Polymerase inhibitor (PARPi), due to Defective Homologous Recombination Repair. 2011;ASCO abstracts:

134 Overgaard J. Hypoxic radiosensitization: adored and ignored. J Clin Oncol 2007;25:4066-74.

135 Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. HPV-associated p16-expression and response to hypoxic modification of radiotherapy in head and neck cancer. Radiother Oncol 2010;94:30-5.

136 Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol 2010;28:4142-8.

137 Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong A et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 2009;74:553-61.

138 Toustrup K, Sorensen BS, Lassen P, Wiuf C, Alsner J, Overgaard J. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. Radiother Oncol 2012;102:122-9.

139 Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 2010;40:294-309.

140 Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38-47.

141 Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J Clin Oncol 2009;27:1992-8.

142 Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. J Clin Oncol 2006;24:2606-11.

143 Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J et al. Molecular classification identifies a subset of human papillomavirus--associated oropharyngeal cancers with favorable prognosis. J Clin Oncol 2006;24:736-47.

144 Lewis JS, Jr., Thorstad WL, Chernock RD, Haughey BH, Yip JH, Zhang Q et al. p16 positive oropharyngeal squamous cell carcinoma:an entity with a favorable prognosis regardless of tumor HPV status. Am J Surg Pathol 2010;34:1088-96.

145 Vincenzi B, Zoccoli A, Pantano F, Venditti O, Galluzzo S. Cetuximab: from bench to bedside. Curr Cancer Drug Targets 2010;10:80-95.

146 Echeverri CJ, Perrimon N. High-throughput RNAi screening in cultured cells: a user's guide. Nat Rev Genet 2006;7:373-84.

147 Smeets MF, Mooren EH, Abdel-Wahab AH, Bartelink H, Begg AC. Differential repair of radiation-induced DNA damage in cells of human squamous cell carcinoma and the effect of caffeine and cysteamine on induction and repair of DNA double-strand breaks. Radiat Res 1994;140:153-60.

148 Weichselbaum RR, Dahlberg W, Beckett M, Karrison T, Miller D, Clark J et al. Radiation-resistant and repair-proficient human tumor cells may be associated with radiotherapy failure in head- and neck-cancer patients. Proc Natl Acad Sci U S A 1986;83:2684-8.

149 Shibuya M. Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). Int J Biochem Cell Biol 2001;33:409-20.

150 Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Nat Rev Cancer 2008;8:942-56.

151 Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A 1998;95:9349-54.

152 Hiratsuka S, Maru Y, Okada A, Seiki M, Noda T, Shibuya M. Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. Cancer Res 2001;61:1207-13.

153 Fan F, Wey JS, McCarty MF, Belcheva A, Liu W, Bauer TW et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. Oncogene 2005;24:2647-53.

154 Lee TH, Seng S, Sekine M, Hinton C, Fu Y, Avraham HK et al. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. PLoS Med 2007;4:e186.

155 Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell 2010;140:268-79.

156 Naik S, Dothager RS, Marasa J, Lewis CL, Piwnica-Worms D. Vascular Endothelial Growth Factor Receptor-1 Is Synthetic Lethal to Aberrant {beta}-Catenin Activation in Colon Cancer. Clin Cancer Res 2009;15:7529-37.

157 De SH, Kimpe M, Isebaert S, Nuyts S. A systematic assessment of radiation dose enhancement by 5-Aza-2'-deoxycytidine and histone deacetylase inhibitors in head-and-neck squamous cell carcinoma. Int J Radiat Oncol Biol Phys 2009;73:904-12.

158 Hu-Lowe DD, Zou HY, Grazzini ML, Hallin ME, Wickman GR, Amundson K et al. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. Clin Cancer Res 2008;14:7272-83.

159 Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT et al. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008;26:127-32.

160 Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. Angiogenesis 2004;7:335-45.

161 Bae DG, Kim TD, Li G, Yoon WH, Chae CB. Anti-flt1 peptide, a vascular endothelial growth factor receptor 1-specific hexapeptide, inhibits tumor growth and metastasis. Clin Cancer Res 2005;11:2651-61.

162 de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995;76 (Pt 4):1057-62.

163 White JS, Weissfeld JL, Ragin CC, Rossie KM, Martin CL, Shuster M et al. The influence of clinical and demographic risk factors on the establishment of head and neck squamous cell carcinoma cell lines. Oral Oncol 2007;43:701-12.

164 Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 2006;124:1283-98.

165 Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 2007;121:2465-72.

166 Rietbergen MM, Leemans CR, Bloemena E, Heideman DA, Braakhuis BJ, Hesselink AT et al. Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by a validated test algorithm. Int J Cancer 2013;132:1565-71.

167 Zhang J, Lu A, Li L, Yue J, Lu Y. p16 Modulates VEGF expression via its interaction with HIF-1alpha in breast cancer cells. Cancer Invest 2010;28:588-97.

168 Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 1996;41:31-9.

169 Tang X, Zhang Q, Nishitani J, Brown J, Shi S, Le AD. Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxia-inducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. Clin Cancer Res 2007;13:2568-76.

170 Fei J, Hong A, Dobbins TA, Jones D, Lee CS, Loo C et al. Prognostic significance of vascular endothelial growth factor in squamous cell carcinomas of the tonsil in relation to human papillomavirus status and epidermal growth factor receptor. Ann Surg Oncol 2009;16:2908-17.

171 Moeller BJ, Yordy JS, Williams MD, Giri U, Raju U, Molkentine DP et al. DNA repair biomarker profiling of head and neck cancer: Ku80 expression predicts locoregional failure and death following radiotherapy. Clin Cancer Res 2011;17:2035-43.

172 Hafkamp HC, Speel EJ, Haesevoets A, Bot FJ, Dinjens WN, Ramaekers FC et al. A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5-8. Int J Cancer 2003;107:394-400.

173 Fouret P, Monceaux G, Temam S, Lacourreye L, St Guily JL. Human papillomavirus in head and neck squamous cell carcinomas in nonsmokers. Arch Otolaryngol Head Neck Surg 1997;123:513-6.

174 Hafkamp HC, Manni JJ, Haesevoets A, Voogd AC, Schepers M, Bot FJ et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. Int J Cancer 2008;122:2656-64.

175 Pignon JP, le MA, Maillard E, Bourhis J. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol 2009;92:4-14.

176 O'Sullivan B, Huang SH, Perez-Ordonez B, Massey C, Siu LL, Weinreb I et al. Outcomes of HPV-related oropharyngeal cancer patients treated by radiotherapy alone using altered fractionation. Radiother Oncol 2012;103:49-56.

177 Dowsett M, Cooke T, Ellis I, Gullick WJ, Gusterson B, Mallon E et al. Assessment of HER2 status in breast cancer: why, when and how? Eur J Cancer 2000;36:170-6.

178 Penault-Llorca F, Adelaide J, Houvenaeghel G, Hassoun J, Birnbaum D, Jacquemier J. Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. J Pathol 1994;173:65-75.

179 Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. J Natl Cancer Inst 1996;88:1054-9.

180 Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 2005;23:1803-10.

181 Hecht JR, Mitchell E, Neubauer MA, Burris HA, III, Swanson P, Lopez T et al. Lack of correlation between epidermal growth factor receptor status and response to Panitumumab monotherapy in metastatic colorectal cancer. Clin Cancer Res 2010;16:2205-13.

182 Fujita K, Sano D, Kimura M, Yamashita Y, Kawakami M, Ishiguro Y et al. Anti-tumor effects of bevacizumab in combination with paclitaxel on head and neck squamous cell carcinoma. Oncol Rep 2007;18:47-51.

183 Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D et al. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev 2011;91:1071-121.

184 Sano D, Kawakami M, Fujita K, Kimura M, Yamashita Y, Ishiguro Y et al. Antitumor effects of ZD6474 on head and neck squamous cell carcinoma. Oncol Rep 2007;17:289-95.

185 Simon R, Maitournam A. Evaluating the efficiency of targeted designs for randomized clinical trials. Clin Cancer Res 2004;10:6759-63.

186 Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. J Clin Oncol 2004;22:777-84.

187 Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. J Clin Oncol 2004;22:785-94.

188 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129-39.

189 Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947-57.

190 Lee JH, Choi S, Lee Y, Lee HJ, Kim KH, Ahn KS et al. Herbal compound farnesiferol C exerts antiangiogenic and antitumor activity and targets multiple aspects of VEGFR1 (Flt1) or VEGFR2 (Flk1) signaling cascades. Mol Cancer Ther 2010;9:389-99.

191 Galkin A, Fallarero A, Vuorela PM. Coumarins permeability in Caco-2 cell model. J Pharm Pharmacol 2009;61:177-84.

192 Shin MC, Zhang J, Min KA, Lee K, Byun Y, David AE et al. Cell-penetrating peptides: Achievements and challenges in application for cancer treatment. J Biomed Mater Res A 2013;

193 Jia F, Liu X, Li L, Mallapragada S, Narasimhan B, Wang Q. Multifunctional nanoparticles for targeted delivery of immune activating and cancer therapeutic agents. J Control Release 2013;

194 Lai SY, Koppikar P, Thomas SM, Childs EE, Egloff AM, Seethala RR et al. Intratumoral epidermal growth factor receptor antisense DNA therapy in head and neck cancer: first human application and potential antitumor mechanisms. J Clin Oncol 2009;27:1235-42.

195 Ni X, Zhang Y, Ribas J, Chowdhury WH, Castanares M, Zhang Z et al. Prostate-targeted radiosensitization via aptamer-shRNA chimeras in human tumor xenografts. J Clin Invest 2011;121:2383-90.

196 Kozielski KL, Tzeng SY, Green JJ. Bioengineered nanoparticles for siRNA delivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2013;5:449-68.

197 Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995;376:66-70.

198 Lee CH, Yen CY, Liu SY, Chen CK, Chiang CF, Shiah SG et al. Axl is a prognostic marker in oral squamous cell carcinoma. Ann Surg Oncol 2012;19 Suppl 3:S500-S508.

199 Dai B, Chen H, Guo S, Yang X, Linn DE, Sun F et al. Compensatory upregulation of tyrosine kinase Etk/BMX in response to androgen deprivation promotes castration-resistant growth of prostate cancer cells. Cancer Res 2010;70:5587-96.

200 Guryanova OA, Wu Q, Cheng L, Lathia JD, Huang Z, Yang J et al. Nonreceptor tyrosine kinase BMX maintains self-renewal and tumorigenic potential of glioblastoma stem cells by activating STAT3. Cancer Cell 2011;19:498-511.

201 Guo S, Sun F, Guo Z, Li W, Alfano A, Chen H et al. Tyrosine kinase ETK/BMX is up-regulated in bladder cancer and predicts poor prognosis in patients with cystectomy. PLoS One 2011;6:e17778.

202 Yarbrough WG, Whigham A, Brown B, Roach M, Slebos R. Phosphoinositide kinase-3 status associated with presence or absence of human papillomavirus in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 2007;69:S98-101.

203 Sorensen BS, Busk M, Olthof N, Speel EJ, Horsman MR, Alsner J et al. Radiosensitivity and effect of hypoxia in HPV positive head and neck cancer cells. Radiother Oncol 2013;

204 Meijer TW, Kaanders JH, Span PN, Bussink J. Targeting hypoxia, HIF-1, and tumor glucose metabolism to improve radiotherapy efficacy. Clin Cancer Res 2012;18:5585-94.

205 Bosco MC, Pierobon D, Blengio F, Raggi F, Vanni C, Gattorno M et al. Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mature dendritic cells: identification of TREM-1 as a novel hypoxic marker in vitro and in vivo. Blood 2011;117:2625-39.

206 Olin MR, Andersen BM, Litterman AJ, Grogan PT, Sarver AL, Robertson PT et al. Oxygen is a master regulator of the immunogenicity of primary human glioma cells. Cancer Res 2011;71:6583-9.

207 Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. HPV-associated p16-expression and response to hypoxic modification of radiotherapy in head and neck cancer. Radiother Oncol 2010;94:30-5.

208 Green JA, Kirwan JM, Tierney JF, Symonds P, Fresco L, Collingwood M et al. Survival and recurrence after concomitant chemotherapy and radiotherapy for cancer of the uterine cervix: a systematic review and meta-analysis. Lancet 2001;358:781-6.

Summary

Head-and-neck squamous cell carcinoma (HNSCC) is the fifth most common malignancy worldwide, responsible for approximately half a million new cases every year. The treatment of this disease is challenging and characterized by high rates of therapy failure and toxicity, stressing the need for new innovative treatment strategies.

In this work we primarily aimed to identify potential starting points for new therapeutic targeting strategies. We therefore performed a high throughput shRNAmir based screen on HNSCC cells with the aim to identify tyrosine kinases that are mediating radiotherapy resistance. Using this screen setup we identified the receptor tyrosine kinase FLT1 (VEGFR1) as an important driver of cell survival and radioresistance, and demonstrate receptor activation through autocrine production of FLT1 ligands. Immunohistochemistry on HNSCC patient samples demonstrated FLT1 and ligands to be uniformly expressed. Interestingly, FLT1 was selectively overexpressed in tumour tissue as compared to non-cancerous epithelium. Remarkably, we found only membrane permeable FLT1 kinase inhibitors to be effective, which was in agreement with the intracellular localization of FLT1.

Taken together, we document expression of FLT1 in HNSCC and demonstrate this kinase to modulate radioresistance and cancer cell survival. Given the fact that FLT1 kinase is selectively upregulated in tumour tissue and that its kinase function seems expendable for normal life and development, this kinase holds great promise as a new potential therapeutic target. Nonetheless several scientific questions still need to be resolved before FLT1 targeted therapy can be transferred from bench to bedside. Most crucial is the development of a good FLT1 targeting drug, which is able to shut down FLT1 activity at concentrations which are maintainable in a clinical setting. Also further validation of such a FLT1 targeting strategy is recommended in different patient derived HNSCC xenograft models to evaluate therapy responses as well as to validate prognostic biomarkers like for example (semi)quantitative VEGFA expression or Human Papilloma Virus (HPV) status.

A subgroup of HNSCC is etiologically related to HPV infection. These HPV related tumors present a different clinical behavior as compared to classical HPV unrelated HNSCC which seems rather paradoxical: they tend to have a better prognosis and show a better response to radio/chemotherapy although these tumors are undifferentiated and show more advanced clinical stages. Additionally in contrast to HPV unrelated HNSCC they seem not responsive to hypoxia targeted therapy although the level of hypoxia between these tumors is comparable. To date these characteristics are unexplained on a molecular level. As a starting point to shed light on this intriguing radiobiologic behavior, we started from observations done on breast cancer cells where it was shown that p16 suppresses the expression of VEGFA (a FLT1 activating ligand), through inhibition of HIF1α, the key regulator of hypoxic metabolism. If we could show that p16 is suppressing HIF1α in HPV positive HNSCC, a resulting reduction of VEGFA expression could maybe explain part of the increased radiosensitivity through impaired FLT1 activity. Additionally, an impaired HIF1α function could lead to hypoxia intolerance, owing to impaired adaptation to cellular hypoxia related metabolic stress. This hypoxia intolerance could then severely impact cellular survival under oxygen deprivation, and therefore lead to increased radiosensitivity *in vivo,* since these cells would be unable to rely on hypoxic niches within a tumor to protect them from radiation. We clearly demonstrated p16 related HIF1α suppressive activity in HPV positive HNSCC resulting in suppressed VEGFA expression. However we also showed that this suppressive activity did not suffice to prevent a HIF1α mediated metabolic shift nor hypoxia intolerance in these cells. The reason why HPV positive HNSCC is not responsive on hypoxia targeted therapy therefore remains elusive. Recent work, however, by the DAHANCA group shows HPV positive HNSCC cells are sensitive to hypoxia targeting but only *in vitro*. It is therefore possible that the true explanation of this strange radiobiological behavior might come from studies investigating the tumor microenvironment like the tumor vasculature or the immune system.

HPV associated HNSCC is currently becoming a global epidemic. According to American SEER data, the incidence of this type of cancer is increasing with 2-3% each year. American studies have shown that 40 to 65% of HNSCC arising from the base of tongue or tonsil could be attributed to HPV16. As already mentioned these tumors are more radiocurable but are not responsive to hypoxia targeted therapy. These factors could probably have an impact on treatment selection. Currently, locally advanced stage oropharyngeal cancers are being treated in the same way as the classical HNSCC with radio-chemotherapy schedules. These intense treatment regimens come however at the cost of severe acute and late toxicity (like dysphagia and PEG-tube dependency). Patients with HPV-associated HNSCC might not need these intense treatment regimens, and perhaps more function sparing therapies lead to equal survival numbers in this population. To set up new clinical studies, we need to have an idea about the prevalence of HPV in oropharyngeal cancer in our patient population in Flanders (Belgium), certainly because large geographical differences seem to exist. Therefore a multicenter cooperative study was undertaken between the radiation-oncology departments of the Flemish universities. We found indeed an increasing incidence of oropharyngeal carcinoma increases in males as well as females. The prevalence of HPV(+) oropharyngeal carcinoma was found to be 24.78% (19.93-30.36%). HPV status remained a strong predictor of better locoregional control after multivariate analysis. Regarding locoregional control we found the addition of concurrent chemotherapy to be of equal benefit in HPV(+) and HPV(-) patients suggesting that treatment de-escalation by omitting concurrent chemotherapy is probably not a good idea.

Samenvatting

Hoofd- en halskanker (HHK) is de vijfde meest voorkomende kanker wereldwijd. Elk jaar wordt de diagnose bij een half miljoen mensen gesteld. Radiotherapie en heelkunde zijn de voornaamste behandelingsmodaliteiten. De behandeling van deze tumoren is ~~een~~ complex en wordt gekenmerkt door belangrijke toxiciteit. Desondanks blijven de genezingskansen beperkt. Nieuwe ~~behandelingstrategieën~~behandelingsstrategieën zijn hierom zeer welkom, zeker indien ze de therapeutische slaagkansen kunnen verhogen zonder meer toxiciteit te veroorzaken.

In dit doctoraal proefschrift werd gepoogd nieuwe aanknopingspunten te identificeren om de effectiviteit van een bestralingsbehandeling te verhogen. Met een RNA-interference gebaseerde screeningstechniek werd de ganse tyrosine kinase familie onderzocht naar proteïnes die de resistentie aan radiotherapie verhogen in HHK ~~cel lijnen~~cellijnen. Met deze setup identificeerden we het FLT1 eiwit dat niet enkel radioresistentie verhoogt maar ook de celgroei stimuleert. Met western blot technieken en ~~quantitatieve~~kwantitatieve PCR konden we inderdaad de expressie en activatie van dit eiwit confirmeren in deze kanker cellen. De activatie van dit receptor eiwit leek niet veroorzaakt te zijn door activerende mutaties, maar door productie van activerende liganden (zoals VEGFA), door de kankercellen zelf. Bijkomend onderzoek op tumor biopsies van HHK patiënten bevestigde verder dat dit FLT1-eiwit (en ligand) door bijna alle HHK's tot expressie wordt gebracht. Een zeer interessante observatie was dat FLT1 selectief tot overexpressie werd gebracht in HHK cellen. Verder werd eerder reeds aangetoond dat de kinase functie van dit eiwit niet essentieel is voor de normale weefselgroei en homeostase. Hierom verwachten we weinig toxiciteit van FLT1 gerichte therapie.

Bij evaluatie van verschillende strategieën om de FLT1 kinase functie stil te leggen noteerden we dat enkel cel membraan permeabele farmaca effectief bleken. De oorzaak hiervan was dat het FLT1 eiwit zich, tegen de verwachtingen in, niet op de

celmembraan bevond, maar wel intracellulair. Er dienen echter nog verscheidene horden genomen te worden voordat FLT1 gerichte therapie in klinische studies op patiënten kan worden getest. Meest essentieel hierbij is de ontwikkeling van een goede en selectieve FLT1 inhibitor, die FLT1 kan uitschakelen aan concentraties die klinisch haalbaar zijn in patiënten.

Een subgroep van HHK wordten geïnduceerd door een infectie met het HPV virus. Deze tumoren, die gekenmerkt worden door p16 overexpressie, hebben een intrigerend radiobiologisch gedrag: hHoewel deze tumoren slecht gedifferentieerd zijn en zich meestal in meer geavanceerde stadia presenteren, hebben ze toch een beduidend betere prognose na radio(chemo)therapie. Bijkomend zien we ook dat deze tumoren, in tegenstelling tot contrast met de klassieke HHK's, niet responsief zijn voor hypoxie gerichte therapie. Nochtans werd reeds aangetoond dat deze beide groepen van tumoren gelijke hoeveelheden van hypoxie vertonen. In de hoop dit eigenaardige gedrag te kunnen verklaren vertrokken we van eerder gedane observaties in borstkanker cellen, waar werd getoond dat p16 de aanmaak van FLT1 activerend VEGFA kan verminderen door de werking van HIF1α te onderdrukken. HIF1α speelt een sleutelrol in de coördinatie van de metabole processen die een cel in staat moeten stellen om te kunnen overleven bij zuurstofgebrek. Hierom leek het mogelijk dat p16/HPV positieve HHK cellen niet zouden kunnen overleven in hypoxische omstandigheden. Mogelijks wordt er hierom geen nut gezien van hypoxie gerichte therapie. Bijkomend zou dit proces ook de verhoogde radiosensitiviteit kunnen verklaren van HPV positieve tumoren: het werd reeds lang aangetoond dat hypoxie tumorcellen beschermt tegen de toxische effecten van bestraling. Met p16 knockdown experimenten konden we aantonen dat p16 de normale werking van HIF1α ondermijnt in HPV positieve HHK cellen. Verder vonden we ook dat in tumorbiopties de van p16/HPV positieve HHK's de maximale VEGFA expressie ook verlaagd was. Deze data suggereren dus inderdaad dat een verminderde FLT1 activatie door gebrek aan VEGFA mogelijks aan de basis ligt van de toegenomen radiogevoeligheid van deze HPV geassocieerde tumoren. We toonden echter ook aan

dat p16 overexpressie de werking van HIF1α onvoldoende kan onderdrukken om hypoxie intolerantie te veroorzaken. De reden waarom HPV positieve tumoren niet responsief zijn aan hypoxie gerichte therapie blijft hierom nog steeds onbekend.

HPV infecties geven momenteel aanleiding tot een wereldwijde epidemie van HHK's gelokaliseerd in de oropharynx. Gegevens uit de VS tonen een toename van dit kankertype met jaarlijks 2-3%. Amerikaanse studies tonen verder ook dat momenteel reeds 2/3 van de HHK's ter hoogte van de tonsillen of de tongbasis door dit virus worden veroorzaakt. Zoals hoger beschreven zijn deze tumoren gevoeliger aan radio(chemo)therapie dan de klassieke HHK's die door alcoholgebruik en roken worden veroorzaakt, maar ze zijn niet gevoelig aan hypoxie gerichte therapie. Deze factoren zullen mogelijk in de toekomst een impact hebben op de selectie van therapie. Momenteel worden de ~~locaal~~lokaal ~~geavanceerde~~ gevorderde HPV geassocieerde HHK's van de oropharynx net zoals de klassieke HHK's behandeld met radiochemotherapie. Deze intensieve behandelingsschema's geven echter aanleiding tot belangrijke acute en laattijdige toxiciteit (zoals dysfagie en soms levenslange afhankelijkheid van sondevoeding). Patiënten met een HPV gerelateerde HHK hebben mogelijks geen nood aan dergelijke intensieve therapie. Het is mogelijk dat minder intensieve behandelingen minder toxiciteit veroorzaken maar toch even efficiënt zijn naar genezingskansen toe. Echter om studies te kunnen opstarten die deze vraagstellingen onderzoeken is het nodig een goed idee te hebben van de huidige prevalentie van HPV geassocieerde HHK tumoren in onze regio (Vlaanderen/België). Er waren echter geen gegevens hieromtrent. Epidemiologische studies toonden verder ook een zeer grote geografische variabiliteit aan, waardoor het moeilijk is een extrapolatie te maken vanuit gegevens uit de ons omringende landen. Hierom werd er een multi-centrische studie opgezet tussen de universitaire radiotherapie centra uit Vlaanderen ~~in samenwerking met het nationale kanker register~~. De gevonden prevalentie van HPV geassocieerde oropharynx carcinomen was 24.78% (19.93 - 30.36%). We noteerden verder ook een toenemende incidentie van oropharynx carcinomen in Vlaanderen bij mannen zowel als vrouwen. HPV status bleek een

sterke predictor te zijn van locoregionale tumor controle na radiotherapie in multivariaat analyse. Met betrekking tot locoregionale tumor controle vonden we ook dat het toevoegen van chemotherapie de genezingskansen verbeterde in zowel HPV positieve als negatieve oropharynx carcinomen. Hierom lijkt ons het weglaten van concurrente chemotherapie geen ideale manier om de toxiciteit van een bestralingsbehandeling te verminderen bij patiënten met een HPV geassocieerde tumor.

Curriculum Vitae

Personal information:

Name	Evert Jan Van Limbergen
Adress	Kapucijnenvoer 94, 3000 Leuven
Telefone	+32.472.86.49.72
Date of birth	03/07/1982
Place of birth	Leuven
E-mail	evert.vanlimbergen@gmail.be
Nationality	Belgian
Marital Status	Married, 2 Children

Education

2001-2003	Bachelor degree Medicine, Catholic university of Leuven (KUL), Magna Cum Laude
2003-2007	Master degree Medicine, Catholic University of Leuven (KUL), Magna Cum Laude
2007-Current	Specialist training towards Radiation Oncologist, Catholic university of Leuven (KUL)
2008-Current:	Doctoral Training Cancer program~~me~~ at the Catholic University of Leuven (KUL)

Professional Experience

2007-2008	Resident at the department of o~~O~~ncology at 2Alice, Uccle, Brussels. Patient care in French speaking environment.
2008-2012	Research fellow of the research foundation of Flanders (FWO aspirant) at the lab of Experimental Radiotherapy (ExpRT). **Subject:** Molecular pathogenesis of head and neck cancer: the role of tyrosine kinases and HPV
2012-current	Resident at the department of radiation-oncology at UH Leuven, Leuven.

International awards

2011	Wolfsberg Travel Award -Wolfsberg Radiobiology meeting ESTRO Ermatingen, Switzerland

2011	Best Poster award ENASCO meeting on molecular markers, Brussels, Belgium

Teaching

2010-2011	Supervisor of undergraduate student: P16INK4a in oropharyngeal cancer: Is the surrogate marker the master switch? Ruveyda Dok,
2010	Lector "Fundamental Aspects of Cancer", Academy/University of Brussels (HUB). Postgratuate radiotherapy technicians.
2011	Lector "Normal tissue effects of ionizing radiation", Catholic University of Leuven (KUL), postgraduate medical radiation ~~phycisists~~physicists, radiotherapist-oncologists in training and 2nd master biomedical sciences.

Met opmaak: Tekstkleur: Auto

Met opmaak: Tekstkleur: Auto

Contribution to writing of research grants with funding obtained

2011	Research fund Anhaive Cancer, King Baudouin Foundation **Project:** Role of Human Papilloma Virus in Head and Neck Cancer: a new player with a potential role in epidemiology, prognosis, prevention and therapy.
2012	Research fund of the Research Foundation Flanders (FWO). **Project**: P16 in HPV related Head and Neck Carcinoma: Taken for a surrogate marker, being the radiobiological master switch?"

Further training and workshops

2008	ESTRO course on Radiobiology (St.Petersburg, Russia)
2008-2009	Biostatistics, Catholic University of Leuven (KUL)
2008-2009	Management: Managing my PhD, Catholic University of Leuven (KUL)
2009	ESTRO course on Molecular Biology for the Radiation Oncologist (Santorini, Greece)
2009	Communication: Poster presentations
2009-2010	Laboratory animal science (module I), Catholic University of Leuven (KUL)

Bibliography

International peer reviewed papers

EJ. Van Limbergen, P. Zabrocki, M. Porcu, E. Hauben, J. Cools, S. Nuyts. FLT1 kinase is a mediator of radioresistance and survival in Head and Neck Squamous Cell Carcinoma. *Acta Oncologica, 2013; Early Online 1-9.*

EJ. Van Limbergen, E. Hauben, F. Duprez, D. Van den Weyngaert, M. Voordeckers, W. DeNeve, S. Nuyts. HPV related Oropharyngeal cancers in Flanders (Belgium): Results from a large multicenter study. *Manuscript submitted to B-ENT, in press*

R. Dok, P. Kalev, **EJ. Van Limbergen**, I. Vázquez, E. Hauben, A. Sablina , S. Nuyts. p16/INK4A impairs homologous recombination-mediated DNA repair in human papillomavirus (HPV) positive-head and neck tumors. *Cancer Research, in revision*

EJ Van Limbergen, R. Dok, S. Nuyts. The molecular basis for the improved radiocurability of HPV associated Head and Neck Cancer: a review of clinical en preclinical evidence. *In preparation*

Abstracts

EJ. Van Limbergen, E. Hauben, S. Nuyts. Role of p16/INK4a as a prognostic factor for radiotherapy response in oropharyngeal carcinoma. BACR meeting, Leuven, Belgium, 30/01/2010. Belg J Med Oncol 2010;4:83-102. *Poster presentation.*

EJ. Van Limbergen, E. Hauben, S. Nuyts. The predictive value of p16 immunohistochemistry in irradiated oropharyngeal carcinoma and its relation with human papilloma virus. ESTRO 29, Barcelona, Spain, 12-16/09/2010. Radiother Oncol 2010;96(Suppl 1):S332. I.F.: 4.343. *Poster presentation.*

EJ. Van Limbergen, E. Hauben, S. Nuyts. HPV and p16 expression as markers for radiation response in oropharyngeal cancer. NWHHT, Maastricht, the Netherlands, 15/10/2010. *Oral presentation.*

EJ. Van Limbergen, R. Dok, E. Hauben, S. Nuyts. P16 immunohistochemistry and HPV-PCR for response prediction after radiotherapy in HNSCC. 3rd ICHNO conference, Barcelona, Spain, 26/02/2011. *Oral presentation by Nuyts S.*

EJ. Van Limbergen, P. Zabrocki, Michaël Porcu, E. Hauben, J. Cools, S. Nuyts. An unbiased shRNA based lentiviral screen identifies tyrosine kinases that are important for radioresistance and survival

in HNSCC. Wolfsberg Radiobiology meeting ESTRO, Ermatingen, Switzerland, 25-27/06/2011. *Poster presentation*

M. Lambrecht, V. Vandecaveye**, EJ. Van Limbergen**, E. Hauben, I. Roebben, R. Dok, F. De Keyzer, R. Hermans, S. Nuyts. The prognostic value of pretherapeutic diffusion weighted MRI in oropharyngeal carcinoma treated with (chemo-)radiotherapy. ICIS 2011, Copenhagen, Denmark 3-5/10/2011.

EJ. Van Limbergen, P. Zabrocki, M. Porcu, E. Hauben, J. Cools, S. Nuyts. An unbiased shRNA based lentiviral screen identifies tyrosine kinases that are important for survival and radioresistance in Head and Neck Squamous Cell Carcinoma. ENASCO meeting, Brussels, Belgium, 27-29/10/2011. Eur J Cancer, 47(suppl 4):S9. IF 4.944. *Oral presentation.*

EJ. Van Limbergen, R. Dok, E. Hauben, S. Nuyts. P16 in head and neck cancer: a marker of impaired HIF1a function and ~~decreased~~decreased hypoxia ~~tollerance~~tolerance? ESTRO31, 2012 May, Barcelona, Spain . *Oral presentation.*

Reviews for F1000

Intratumoral Epidermal Growth Factor Receptor Antisense DNA therapy in Head and Neck Cancer: First Human Application and Potential Antitumor Mechanisms. Lai SY, et al. Reviewed with S. Nuyts for **F1000** (September 2009).

HPV-associated p16-expression and response to hypoxic modification of radiotherapy in head and neck cancer. P Lassen, et al. Radiotherapy and Oncology 2009. Reviewed with S. Nuyts for **F1000** (January 2010).

Receptor Tyrosine kinase coactivation networks in cancer. AM Xu and PH. Huang Cancer Research 2010. Reviewed with S. Nuyts **voor F1000** (May 2010).